

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

AD-A212 666

ELECTED
25-1989

1b RESTRICTIVE MARKINGS

NA

3 DISTRIBUTION / AVAILABILITY OF REPORT

Distribution Unlimited

4 PERFORMING ORGANIZATION REPORT NUMBER(S)

5 MONITORING ORGANIZATION REPORT NUMBER(S)

NA

6a NAME OF PERFORMING ORGANIZATION
University of California,
Los Angeles6b OFFICE SYMBOL
(If applicable)7a NAME OF MONITORING ORGANIZATION
Office of Naval Research6c ADDRESS (City, State, and ZIP Code)
405 Hilgard
Los Angeles, CA 900247b ADDRESS (City, State, and ZIP Code)
800 N. Quincy St.
Arlington, VA 22217-50008a NAME OF FUNDING / SPONSORING
ORGANIZATION
Office of Naval Research8b OFFICE SYMBOL
(If applicable)
ONR9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
N00014-88-J-11338c ADDRESS (City, State, and ZIP Code)
800 N. Quincy St.
Arlington, VA 22217-5000

10 SOURCE OF FUNDING NUMBERS

PROGRAM
ELEMENT NO
61153NPROJECT
NO
RRO 4106TASK
NO
4412041/02WORK UNIT
ACCESSION NO

11 TITLE (Include Security Classification)

(U) Training Program in Marine Molecular Biology

12 PERSONAL AUTHOR(S)

David J. Chapman

13a TYPE OF REPORT
Annual13b TIME COVERED
FROM 1988 TO 198914 DATE OF REPORT (Year, Month, Day)
1989 Sept 1.

15 PAGE COUNT

16 SUPPLEMENTARY NOTATION

17 COSATI CODES

FIELD GROUP SUB-GROUP

18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)

Training program; marine molecular biology; graduate.

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The Marine Molecular Biology Training Program was established with the specific aim of introducing and training graduate students, who themselves are trained marine biologists, to the techniques of modern molecular biologists.

The 10 week course was taught at the USC Marine Station at Santa Catalina Isl. April-June 1989. Twelve students, from 7 institutions, chosen from a nationwide applicant pool were selected, and requested at the faculty. Four faculty were involved: Professor C. Brunk (Molecular Biology); Professor E. Gonzalez (Cell and Molecular Biology); Professor L. Muscatine and D. Chapman (organismic-physiology). The first two weeks were devoted to instruction in DNA methodology (C. Brunk) and the second to methodology for proteins, enzymes,

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21 ABSTRACT SECURITY CLASSIFICATION

(U)

22a NAME OF RESPONSIBLE INDIVIDUAL
M. Marron22b TELEPHONE (Include Area Code)
202-696-470022c OFFICE SYMBOL
ONR

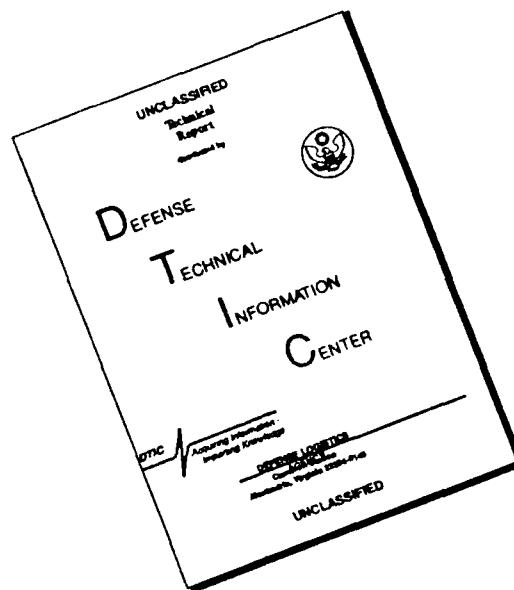
19. ABSTRACT (continued)

RNA and organelles. The syllabi and manual for this 4 week period are attached. The remaining 6 weeks was set aside for individual projects for which the students were required (for grades) to provide a written research report and an oral presentation. Evaluation by the students of the course were solicited and are attached .

As expected a number of logistic problems were inevitable for the first time. These have been identified and will be remedied for the second year.

The course achieved its stated goal and was very well received by the participating students.

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ABSTRACT

The Marine Molecular Biology Training Program was established with the specific aim of introducing and training graduate students, who themselves are trained marine biologists, to the techniques of modern molecular biologists.

The 10 week course was taught at the USC Marine Station at Santa Catalina Isl. April-June 1989. Twelve students, from 7 institutions, chosen from a nationwide applicant pool were selected, and requested at the faculty. Four faculty were involved: Professor C. Brunk (Molecular Biology); Professor E. Gonzalez (Cell and Molecular Biology); Professor L. Muscatine and D. Chapman (organismic-physiology). The first two weeks were devoted to instruction in DNA methodology (C. Brunk) and the second to methodology for proteins, enzymes, RNA and organelles. The syllabi and manual for this 4 week period are attached. The remaining 6 weeks was set aside for individual projects for which the students were required (for grades) to provide a written research report and an oral presentation. Evaluation by the students of the course were solicited and are attached .

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Justification	
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Distribution/	
Availability Codes	
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A-1	20

MARINE MOLECULAR BIOLOGY TRAINING PROGRAM

Annual Report

The objective of this 10 week training program, as outlined in the original proposal, was the introduction of graduate students currently trained in the field of marine biology, to the techniques of modern cell and molecular biology. Marine biology has been slow to apply these techniques for a number of reasons, not the least of which is the severe shortage of practitioners trained in the techniques.

Program: The training course, involving official UCLA course credit (8 units for the research; 4 units for the report and seminar presentation) was held from April 3, 1989 to June 10, 1989 at the marine station of the University of Southern California at Santa Catalina Island.

Student Selection: The program was advertised nationally during Fall in Science and through brochures sent to approximately 250 institutions. Graduate students were chosen from an applicant pool of approximately 30 on the basis of academic performance, applicability of the course to their career goals, and documentation (including letters of reference) of the student's need and qualification for the course. All applicants had to be graduate students and to be majoring in or be trained in some area of marine biological science, since no formal instruction was provided in the area of marine biology in the course. The maximum number of students that can be accommodated in the facilities (research laboratory) is 12 - 14. Twelve students were selected.

University of Rhode Island	1
University of Massachusetts	1
University of Delaware	3
UCLA	3
University of Alaska	1
University of California Santa Cruz	1
Boston University	1
Yale University	1

Students were supported in part by travel and fellowship funds from the ONR training grant.

Equipment and supplies: a full range of necessary equipment was provided by loan from Beckman Instruments and Pharmacia--LKB. These two companies are due a major vote of thanks for their cooperation and generosity. Many individuals from the two companies were involved, but particular thanks go to Tim Stebbins (Beckman), Mike Cammarata and Julie Perinne (Pharmacia--LKB) for their major organizational role. Additional instrument help was provided by Hoefer Instrument. Equipment and supplies not covered by the grant were provided from departmental instructional funds. Additional matching funds to cover the rental charge of the laboratories and the teaching assistant stipends were provided by UCLA.

Instructional Staff:

Professor C. Brunk: techniques appropriate to DNA methodology, cloning, gene libraries;

Professor E. Gonzalez: techniques of cell biology; organelle isolation, mRNA, protein/enzyme purification, gene expression.

Teaching assistants for these two faculty were M. Harmon, L. Sadler, C. Kerfeld. The following technicians M. Brush (Gonzalez), F. Tabatabai (Chapman) and R. Kahn (Brunk) provided additional technical support.

Professors D. Chapman and L. Muscatine provided the appropriate academic support and instruction in the field of Marine Biology.

Academic Program: The first four weeks were set aside for instruction in techniques. A typical format (Monday through Friday) involved a one to two hour background lecture in the morning, with the remainder of the morning, all afternoon and evening set aside for "hands-on" instruction under immediate supervision of the faculty, teaching assistants and research technicians. Saturdays involved more informal instruction and practice. The first two weeks (Professor Brunk) were devoted to DNA techniques using DNA from sea urchin sperm. All the protocols had been pretested in the fall and winter at UCLA. (Appendix 1 lists the schedule for this section.) The second two weeks were devoted to techniques in cell biology, gene expression and macromolecular separation and purification (Professor Gonzalez).

For this section of the course the techniques (again all pretested on campus) were based upon analyses and use of marine algae, particularly Codium fragile. A specially prepared protocol manual was provided for the students. By the end of the fourth week each student selected, of their own choice, a research project in marine molecular biology, involving the local flora or fauna. These projects were approved (or modified as necessary) by the faculty and had to involve original research. At the end of the quarter the students presented the results of their research as a seminar to the entire group (faculty, TAs and students) and were required to provide a full written report, in scientific paper format. The seminar presentation and

their research served as the basis for the two grades. Copies of the reports are enclosed.

Course evaluation: In addition to the student impressions communicated directly at the ONR site visit team (Drs. Newburgh, Marron and Alberte), a written course evaluation was requested of the students. These are enclosed.

Prospectus: The overall consensus of all parties is that the course achieved its objectives. The physical format and location are ideal. The students are sequestered away from the main campus and city distractions, thus providing the incentive for total immersion. The disadvantages lie in the lack of ancillary support facilities that must be remedied by next year: ice machines, adequate refrigeration and freezer; adequate quality distilled water. While the supply of and access to small instruments was superb, major instrumentation, e.g., -70 freezer, ultracentrifuge, computer accessing still remain a problem. These will be remedied. The logistics of providing adequate dry ice, liquid N and journal articles (the USC station library is totally non-existent) are a problem. For the future courses we have developed a good log book of what is needed and how much; it is essential to take everything that the course will need and might conceivably need, since valuable time and momentum is lost when research stalls while waiting for supplies.

This course was the first of its kind ever to be given at the Catalina Marine Station, and the first time the UCLA Marine and Molecular faculty had to come together in this fashion. There were problems; they were to be expected. The experience has been invaluable and we now have a much clearer

idea of what can be done, what should be discouraged and most importantly the logistic problems. These will be resolved by next year and the course will be even better.

TEXTS

- B. Perbal: Practical Guide to Molecular Cloning
- S. Berger & A. Kimmel: Guide to Molecular Cloning Techniques
- M. Schuler & R. Zielinski: Methods in Plant Molecular Biology
- A. Weissbach & H. Weissbach: Methods for Plant Molecular Biology

PROJECTS

1. Microsomal Cytochrome P450 of Marine Polychaetes
2. Heat shock response: Is it observed in intertidal organisms
3. Actin in wounded and intact Codium fragile
4. Metal binding proteins in the rock scallop, Hinnites: Isolation and characterization of the genomic DNA encoding metallothionein.
5. Preliminary attempts at extraction of mitochondrial DNA from the copepod Pleuromamma borealis
6. Molecular phylogeny of the green algal genus Codium using restriction fragment length polymorphism analysis of chloroplast DNA.
7. DNA isolation, hybridization and cloning in sea anemones
8. Characterization of genomic and mitochondrial DNA of the black abalone, Haliotis cracherodii
9. DNA characterization of the ctenophore, Leucothea pulchra
10. Detection of the Mn superoxide dismutase and catalase genes in the green alga Codium
11. Characterization of mitochondrial DNA from the horn shark Heterodontus francisci
12. Histocompatibility markers in the anemone Anthopleura elegantissima

MARINE MOLECULAR BIOLOGY
CATALINA MARINE SCIENCE CENTER, SPRING 1989

COURSE EVALUATION

In the space below (use additional sheets if necessary) please give your candid opinion of the UCLA Marine Molecular Biology Course.

Overall my impression of the course was fantastic. I liked the idea of splitting the course into a four week instructional section and a six week project section. Also, holding the course on Catalina Island made us a captive audience. Lab time was optimized and we were exposed to many techniques. The donated equipment was impressive. However, I think that a few changes could benefit the course.

1. Students came from very different backgrounds and some did not know how to use micropipetors or were not aware of general lab courtesies. These points need to be stressed.
2. A better, more reliable distilled water system is needed. Perhaps the available water source was not adequate and was the root of some of our problems.
3. The text that we purchased was probably not the best investment. I think that Perbal's manual would have been more useful.
4. Logistics, such as well defined work stations for students, clearly labelled shelves and drawers, labelled perishable items, etc., would have helped avoid some frustration. A lot of time was spent looking for a piece of equipment, a reagent, or frozen samples. MORE GLASSWARE!
5. There was some confusion about the last day of the course. This created a lag period between the end of the course and ferry and/or flight times. Also, it would have been useful for the students to have travel expense forms completed before leaving the island. This way a minimum amount of time would have passed before reimbursement.
6. None of the east coast students knew much about California marine life. It would be beneficial to use the first couple of course days to do some field work. The class could then identify a few interesting animal-plant interactions and other marine life phenomena. All of the independent projects could stem from one major topic. That way it is more likely that a publication would result and less likely that a student would pick a project that he or she was already familiar with. Approximately one third of the class worked on aspects of their thesis projects. I thought that this tended to breed competitiveness and weaken the goals of the course. The main point of a marine molecular training course is to learn and apply the techniques to many types of marine problems.
7. More students would have been able to try more procedures during the molecular techniques section of the course if we had followed through this period using only sea urchin DNA. Too many of the students were hung up too early on isolating DNA from different organisms. I would also liked to have run RNA gels and done Northern analysis. Many of the students mentioned during

their talks that the next step in their projects would have been to look at gene expression. I also think that CsCl gradients are very important tools in molecular biology and should be taught during the first two weeks of this course.

8. I was disappointed that during the protein section of the course we did not make great use of some of the equipment since the class experiments failed. I do realize that optimizing a system for protein (or DNA) extractions for a particular plant or animal is difficult. However, if the experiment is run through beforehand at UCLA, and back up proteins (or DNA, or RNA) are stored frozen, students would have the opportunity to try all the techniques available in the course. A training course to me means learning the techniques. Students have plenty of time to fumble and learn how to optimize a system on their own, through the course independent projects and in their thesis research.

9. I found that the protein section of the course was very valuable. It demonstrated to us how to look at a biological/ecological phenomena and follow it through to the protein level, to the gene expression level, and to the DNA level. It is very useful to approach a problem from a few different ways (ie. through DNA and protein analysis).

10. During this course, one of the most disturbing things that occurred was the favoritism displayed by one of the faculty members for some students. I believe that all of the students worked hard, learned quite a bit, and got results. This caused some bad feelings and it was unnecessary.

11. All of the TA's, especially Mike Brush and Cheryl Kerfeld, were extremely knowledgeable and helpful. I appreciate the difficulties of putting a course of this nature together and I feel that Drs. Gonzalez and Brunk put a great deal of effort into making it a success. I was also impressed that Dr. Chapman was able to spend as much time as he did on this course (algal round tables, dry ice and supply runs, reference gatherings, etc.) although he was very busy. I enjoyed meeting and listening to the guest speakers brought in by Dr. Muscatine. They were all very interesting as well as informative. It is important and encouraging to listen to scientists who are on the forefront of marine molecular biology.

Overall, this course was one of the best learning experiences that I have had and I hope that it continues to be a success.

MARINE MOLECULAR BIOLOGY
CATALINA MARINE SCIENCE CENTER, SPRING 1989

COURSE EVALUATION

In the space below (use additional sheets if necessary) please give your candid opinion of the UCLA Marine Molecular Biology Course.

The Marine Molecular Biology Quarter was a unique and extremely worthwhile course. In terms of knowledge gained, it was the most productive course which I have ever taken. However, as is often the case with something new, a bit of fine tuning is still required to bring this course up to its full potential. But first, let's dwell on the those things which worked well.

The Catalina Marine Science Center was a fine location for this course. In particular, the isolation, though sometimes tedious, provided an opportunity for full immersion into molecular biology with no distractions. Immediate access to a diverse marine flora and fauna provided a continuous source of experimental material. This factor is particularly important for projects encompassing both field and laboratory experiments.

The equipment was first-rate in terms of quality and quantity. The opportunity to get hands-on experience with the equipment was invaluable. The supply of chemicals, reagents, enzymes, etc., was also very good. More glassware would have been helpful, however. I would especially like to commend the efforts which were made to fill special requests. Despite our isolation, there was little we lacked.

The faculty and TA support and enthusiasm were a key part of the success of the course. The opportunity to have questions resolved almost immediately made our time very productive. Bringing in guest lecturers was also very beneficial. Exposure to researchers presently doing marine molecular biology was enlightening both from the aspect of their successes as well as the pitfalls which they have encountered. In addition, demonstrations of unique techniques which these researchers possess might be very useful.

Despite the overall success of the course, there are several things which I think would help to make the course even better. For example, a brief 2-3 day introduction to the flora and fauna of Catalina and the kinds of research questions available would have been time extremely well spent. Many people in the class were not from the West Coast nor had ever been to Catalina. Such an exercise would have been very beneficial to put things into perspective.

Since this was essentially a techniques course, I think that it should have been important that everyone have an opportunity to get hands-on experience with all of the techniques. For this reason, I think it would have been better if our project areas were more defined in terms of working with organisms in which the likelihood of successfully extracting DNA, RNA, or protein is assured. Obviously, when working on our own research it will be necessary to spend time optimizing isolation procedures, but then time

is not as critical as in a ten week course. A possible approach might be to select one animal and one plant species and develop a comprehensive set of research questions around these organisms. Also, I do not think that it is appropriate for students to work on their personal research problem in the context of the course. A course such as this is an opportunity to expand ones' horizons away from ones' main research.

Finally, one item which should be discussed with CMSC is obtaining an improved water system. This was certainly a major concern of everyone in the course. Also, the opportunity to scuba dive, if one is certified, should not be missed. Some mechanism should be setup by which students outside of the UC system can be assured of certification before arriving for the course.

Susan B. Kane
Course Evaluation
July 10, 1989

The UCLA Marine Molecular Biology Course was excellent. Much credit is due to the organizers, teachers and T.A.'s. They gave us a four-de-force of molecular and biochemical skills that will be invaluable to me in my dissertation work and in my future career.

The DNA section of the course was superb. Dr. Brunk and associates were incredibly well organized and prepared. I think that their format worked very well. We had a one hour talk each morning, giving a theoretical overview of the topic at hand, and the logistics of the day's activities. This worked out better than having evening talks because the students were more alert and enthusiastic in the morning. (As an aside, talks that were held after lunch were particularly draining. We were embarrassed when we had trouble concentrating on the talk because we were falling asleep!)

The DNA and RNA experiments were very well prepared. These protocols worked very well. Sometimes the techniques were presented as "demonstrations" that the class watched. In this way, everyone's time was used most effectively.

I think that it is very important to present experiments that have been worked out in advance. Granted, there are no guarantees that even a tried and true technique will always work, especially given the vagaries of "the island". Nonetheless, it really is a more effective use of everyone's time if the bugs are worked out in advance.

I think that some students might have benefitted from continuing on with a well worked out system, such as the sea urchin. Because mitochondrial DNA probes were already available, students could have made some rapid progress with this system. Of course, some of us wanted to work on our "own organisms", which was very worthwhile and valuable. In the future, however, it might be helpful if the teachers emphasize the advantages of working on the system at hand. For example, the student can run through more of the techniques, such as making and screening a library, if they don't have to spend time on the preliminaries.

Along these lines, there should have been better communication before the course started as to what it would entail. I had no idea that we would have six weeks to work on our own projects. Had I known, I would have brought papers, protocols, reagents and samples with me to Catalina. I also would have given more thought to what I might do for a project. If the goal is really to have the students publish, they should be given a head start.

The general emphasis on plant systems did not match the interests of many of the students. For example, the class text was not ideal for the broad range of interests. Copies of Perbal and a good protein chemistry text would have been preferable. Also, several people were disappointed that Dr. Powers was unable to attend since interest in mitochondrial DNA was high.

Library facilities should be improved for next year. Specifically, good texts in biochemistry, protein chemistry and purification and immunoassays would be helpful. A better system

for getting papers from the library journals should be established. Melvyl should be set up at the beginning of the course. A list of UCLA's periodical holdings should be provided. A brief training session on the computer should be done at the outset. Students should be told to bring their own 3 1/2 " disks. Each speaker should provide a reference list that should be combined into a master list at the end of the class. The students themselves could be responsible for preparing this.

To the credit of us all, the ordering of supplies went very well. However, a better system should be worked out for the future. Perhaps responsibilities could be divided among the students.

I was disappointed that there was no formal field component to the class. The Catalina ecosystem is very diverse and interesting. I would have been very interested to have an ecologist speak to us.

Some systems were underutilized, perhaps because demonstrations were inadequate. This was true for the FAST system and the HPLC.

The lab manual put together for the second part of the course was excellent. It would have been helpful to have more of the protocols written out for the first part of the course as well.

I think that the emphasis on publication was unrealistic. This was a training course. We should have been concentrating on learning new techniques. It might have been better if the students felt free to run through all of the protocols available.

rather than restrict themselves to one area.

I hope that these suggestions are useful for the future. Let me say again that this was an excellent course. The T.A.'s and teachers deserve a lot of credit for an outstanding job.



University of Delaware

COLLEGE OF MARINE STUDIES
LEWES COMPLEX
LEWES, DELAWARE 19958-1298

(302) 645-4209

June 20, 1989

Dr. David Chapman
Department of Biology
University of California, Los Angeles
Los Angeles, California 90024

Dear David,

At last, here is the final product! I realize that I'm submitting this a few days late and I hope that I've not inconvenienced you by doing so. Thanks for a great course, I am still trying to sort out everything we learned. If you need any assistance in the future concerning advertising the course just let me know, I'll be happy to do anything that I can to help. I have also enclosed my UCLA travel reimbursement form and a xeroxed copy of my plane ticket; U. of Delaware requires that we submit the original plane ticket even for partial reimbursements. If there is a problem with your business office concerning the xeroxed copy I can probably get the original receipt back from our business office after they process my travel forms. Just let me know if you need the original.

Have a great summer and please be sure to come to Lewes next time you visit the mid-Atlantic area.

Sincerely,

A handwritten signature in cursive script that reads "Karolyn".

Karolyn M. Mueller

MARINE MOLECULAR BIOLOGY
CATALINA MARINE SCIENCE CENTER, SPRING 1989

COURSE EVALUATION

In the space below (use additional sheets if necessary) please give your candid opinion of the UCLA Marine Molecular Biology Course.

As a first-year marine molecular biology graduate student, I found this course challenging and rewarding. In a remote area like Catalina, I was impressed by the UCLA Biology department's effort in providing us full-line of top-notch equipment and adequate supplies of chemicals.

During the first four weeks, I've learned a great deal from professors and guest lecturers. They really help me to understand the course materials and to decide my independent-study topic. The last six weeks of independent research, I wish would have a number of workable project topics to choose. Thus, we would be able to learn more rather than spending five weeks of time to discover topic's unworkable. Due to time constraint and lack of experience in this field, we would have learned more if two or three students work on a pre-set, workable project which covers both molecular biology and cell biology. Thank you for your attention and help. Best wishes!
Have a great summer!

MARINE MOLECULAR BIOLOGY
CATALINA MARINE SCIENCE CENTER, SPRING 1989

COURSE EVALUATION

In the space below (use additional sheets if necessary) please give your candid opinion of the UCLA Marine Molecular Biology Course.

- 1) Good - I was very impressed with the equipment and reagent availability. Technique instruction was very good. Overall I feel this was a great opportunity with more information & techniques available than I had time to assimilate. I also liked the Catalina marine lab and the availability of specimens. The atmosphere was conducive for research; the student selection provided a cohesive yet diverse group of peers.

- 2) Bad - I was surprised at the poor quality of the Catalina library and access to UCLA resources. I also had expected better computer facilities. I didn't get necessary scheduling and travel information in a timely manner. The stated expectations for student performance ranged from merely gaining experience to publication, which caused confusion in planning. This should be a Pass/Fail course.

MARINE MOLECULAR BIOLOGY
CATALINA MARINE SCIENCE CENTER, SPRING 1989

COURSE EVALUATION

In the space below (use additional sheets if necessary) please give your candid opinion of the UCLA Marine Molecular Biology Course..

Organization - pretty good for a pilot program - I'm sure things will go smoother next year.

Faculty - good split into techniques people + organism people

Instruction - good, but could have used more discussion of theory ~~what is mtDNA~~ (pros + cons of mtDNA, options for analysis of data ...)

Speakers - good, but it might have been better if they'd been scheduled earlier. And what happened to Dennis Powers?

Facilities/equipment - Great! I really appreciate all the work that went into setting up the labs. Although it would certainly have been easier to have held it somewhere like UCLA, I think it was more appropriate to hold it at a real marine lab - plus, it gave us ~~a chance~~ practice in improvisation that we'll need in our own labs.

TAs - A+ Very helpful, knowledgeable, + great fun at parties.

MARINE MOLECULAR BIOLOGY
CATALINA MARINE SCIENCE CENTER, SPRING 1989

COURSE EVALUATION

My overall evaluation of the course is that it was excellent; I learned, or had opportunity to learn, most of what I had hoped to gain from it. I was impressed with the magnitude of the effort that went into putting the course together, and with the dedication of the people who instructed and TA'd the course. The following criticisms are offered with the sole purpose of making the course even better next year.

1. Most of the students in the class work with animals and probably indicated this on their application forms. Yet, many of the lectures, invited speakers and even laboratory material centered around marine algae. While I have personally done a fair amount of work with marine algae, have a great interest in it, and enjoyed the opportunity of learning more about it, my main interests lie with colonial marine animals. Thus I felt that the main emphasis of the course would have been more profitably spent, for me and for others, on animal systems. However, I realize that the course had to be planned in advance of any knowledge of the interests of the applicants, and that the content depended to large extent upon the specialties of the instructors involved.

2. Some of the topics advertised in the brochure were not covered at all in the course. These included: growth rate measurement, nutrient analyses, gas fluxes, ultracentrifugation (for all practical purposes), and antibody preparation. I liked Linda Goff's presentation on epifluorescence microscopy, and would have liked to have spent at least a week on that topic (perhaps during the project period).

3. Better computer facilities are a must. There should be at the very least an IBM compatible and an Apple, with printing capabilities. Students should be encouraged to bring software pertinent to their disciplines. This could prove to be a useful exchange of information. I would also have liked exposure to what is available in sequence processing software, gene information banks, etc. This whole branch of current technology was virtually ignored.

4. Access to library materials was limited, and it was sometimes hard to locate or obtain references without actually going to UCLA, a difficult undertaking for a non-resident student. A more organized procedure, perhaps involving an on-campus TA or other paid help, to expedite obtaining references would be a great asset for next year.

5. Everyone involved in the course was aware of the limitations of the physical facilities, especially the lack of on-line vacuum and gas, and the shortage of adequately pure water. I'll just get it on the record once again.

6. In spite of the logistical difficulties in getting necessary reagents and supplies to Catalina in a timely manner, I would be strongly opposed to moving the course to the main campus. The isolation in a natural setting

close to the marine environment was a major advantage. This was a valuable opportunity for students from other parts of the country to learn something of the Pacific nearshore ecosystem. I personally appreciated also the opportunity to learn about the ecology of Catalina Island. The students formed closer bonds with one another and with the instructors and visiting speakers than they would in the city. And a walk in the countryside or occasional blow-out in Two Harbors was a much more amenable interlude to work than hitting the bars in L.A. until 2 am on a regular basis, which would have been likely given the intensity of the course.

7. In several instances there was voluble dissent among some of the instructors about how the course ought to be run. This gave the students, who could not help overhearing, somewhat the feeling of airline passengers who find out that the pilot has just had a heart attack and that the co-pilot is drunk. Whatever their differences, the instructors should present a calm and united front to the students.

8. I would like to commend George Matsumoto and Blaise Eichner, UCLA grad students attending the course, for their unselfish efforts to help the rest of us adjust to life at CMSC. The morning coffee in the tank room, the occasional movie, their knowledge of the local flora and fauna, their assistance to divers, and their roles as shuttle ferry operators assisted all of us. Whenever we needed to know where something was at CMSC, we asked them.

9. Three or four weeks before the end of the course, we were informed that term papers were due one week after we left the island. This was after a number of students had made plans to travel following the course, or had made other serious commitments. Many of us had not been home for two and a half months and had a lot waiting for us when we returned. I don't feel this stringent a deadline was in anyone's best interest, and certainly not in the best interest of quality papers.

10. In planning our travel to and from L.A., there was uncertainty as to exactly when the course would end. Because of this, some students made return reservations 2-4 days after the nominal end of the course and 3-5 days after the effective end. In the future, this ambiguity should be removed, and an explicit (early) date of departure given to students.

11. The entire staff of the Catalina Marine Science Center is to be complimented for their friendliness, helpfulness, and willingness to make our stay workable. Their accomodation came as a pleasant surprise in a world where one expects apathy and abuse.

12. As to the organization of the course, I would be hard put to say how I would do it differently. I felt that the emphasis on obtaining publishable results was naive on the parts of some of the instructors given the time frame, the logistical problems, the non-trivial nature of the work, and the overall intent of the course. However, to strive to produce publishable results was a useful impetus to work hard and produce the best work possible under the circumstances, and I would be hesitant to remove this goal, however unrealistic. I liked working on a problem of my own choosing and design. There is nothing like interest to spur one on. For me, the effective period of working on the project was about 4 weeks, because it

took 2 weeks to crystallize what I wanted to do, order supplies, read, and generally get my act together. What might be more useful next year is 4 weeks of formal instruction, followed by a two week period with about half formal instruction, planning of projects and, depending upon the student, beginning of projects. More concrete guidance by instructors during this period might be advised. One of the great strengths of the course, perhaps not planned, was to have the students working on a variety of problems with a variety of organisms...perhaps not succeeding, but all of us aware of the problems and pitfalls encountered by the others. This was in many ways preferable, from a pedagogical point of view, than having all of us working on the same organism and succeeding at everything we did. After much thought, I'd not change it a whole lot.

Congratulations Cliff, Elma, David, Len, Michael, Margaret, Lori, Fay, Bobby & Cheryl (did I forget anyone?) on a job well done.

Matt Dick

RAST

MARINE MOLECULAR BIOLOGY
CATALINA MARINE SCIENCE CENTER, SPRING 1989

COURSE EVALUATION

In the space below (use additional sheets if necessary) please give your candid opinion of the UCLA Marine Molecular Biology Course.

OVERALL I was very pleased with the course
Having the course on Catalina Both because of its
isolation and the easy ACCESS to marine organisms
was a good idea.

Working with an organism of our own choice during
the projects Both kept our interest up and gave us
A headstart on our research.

It may be a good idea, however, to run two projects
One with our own organism & one with an organism
that is characterized well enough to assure some degree
of success (eg Sea Urchins).

More morning lectures throughout the course delving into
greater detail on the methods we learned would also
have been helpful. Say 1 hr lectures from 8:30 to 9:30
in the morning. I don't think that this would have
interfered with our projects.

Speakers oriented toward population Biology & molecular
methods would have been helpful as many of us were
interested in investigating population structure.

An organized seminar going over current papers using
techniques that we learned would also add to the experience.
Say $\frac{1}{2}$ hr 45 min a night presentation of one paper by a student.

Essentially, I learned from the course most of what I had
hoped to, and I plan to continue the work that I did
for my project.

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MARINE MOLECULAR BIOLOGY
CATALINA MARINE SCIENCE CENTER, SPRING 1989

COURSE EVALUATION

Firstly, I like to express my thanks and appreciation to all the people involved in making the course possible. I am also indebted to the Office of Naval Research for providing me with a fellowship to attend the course. My thanks are also due to Pharmacia LKB Biotechnology, Inc, Beckman Instruments, Inc, Gilson Medical Electronica, Inc and Hoefer Scientific Instruments for their generous gifts and equipment loans without which the course will not be able to run properly.

Below are my candid opinion of the course:

(1) Laboratory manuals/handouts

As this course is designed for those who have no previous experience in molecular biology, well-written laboratory manuals or handouts are absolutely essential to lead the students along. Elma and Mike have done a superb job in preparing a detailed and yet readable manual. Their manual proved to be very useful in helping the students to follow through experiments which involve more than one procedures and/or one day to complete. Also, their manual also proved to be indispensable for independent research.

Cliff and his teaching assistants put in a lot of effort to introduce the subject matter to the students. However, their effort was negated to a great extent by only providing occasional sheets of handouts which often are not well written. Cliff adopted a Julian child approach in running the lab. Given this

format of teaching and without a manual, very soon the students were confused and lost track of what they were doing. Also, the students did not actually know what the teaching assistants have done before they took over the experiment. This effect was felt when the students were working on their independent projects. For example, a student used unequilibrated phenol/chloroform to extract DNA and thus wasted two weeks. Another example, most of us did not know how to prepare 5 X NET* simply because we did not have the recipe at hand. It is true that we were told to take notes in the lab. However, given a laboratory setting and often instructions were given when not everybody was around, our notes turned out to be not very useful for independent research.

If it is redundant to write a manual for the molecular biology part of the course, I suggest using Perbal or the new edition of Maniatis, if it comes out in time, for the next year's course. Otherwise, using photocopies from various sources would also be useful. However, I am of the opinion that it is worth the effort to write our own manual which is specially tailored for marine organisms. Given the time and as our knowledge on marine molecular biology improves, the manual would ultimately be published as a text. If so, this would be a big contribution to marine sciences.

(2) Lectures

As opposed to what was announced, the course assumed a lot of knowledge in molecular biology. Since most of the students in marine sciences do not have much exposure to molecular biology, daily lecture of 60-80 minutes on the theories concerned is essential. Only with an understanding of the theories involved would the students be able to apply the techniques intelligently to their research.

It may be a good idea to require the students to do some solid background readings before attending the course. The book "Molecular Design of Life" by Lubert Stryer (Pub. by W.H. Freeman) is suggested. It is an excellent introduction to the biochemistry of proteins and nucleic acids.

(3) Independent Research

It was wise to allow six weeks for independent research. I learned the most during this period of time.

The DNA group had tremendous problems trying to extract mtDNA and chloroplast DNA. Unfortunately, they did not receive much help mainly because none of the staff was familiar with the bench-top ultracentrifuge. The other reason was that we could not use the fluorescence microscope to run independent assay to find out whether the organelles were successfully extracted before proceeding further with the experiment as the microscope lacked the appropriate lenses. Given a wide range of organisms the students can work on, it might be a good idea to limit the choice to two species each of marine algae and animals. Bottle-neck

problems like extraction of non-genomic DNA or specific organelles of interest should then be solved before those organisms are thrown to the students. In this way, precious time could be saved and used fruitfully to address specific problems of interest. Also, by doing so the students may even combine their findings and hopefully produce some publishable results. This arrangement may also facilitate rounding up relevant reading materials required for the research.

Also, it was not a good idea to allow students to work on their thesis research. In fact, some students spent the last week extracting DNA for later use instead of proceeding with their research as planned. This was a loss to the whole class for less material was presented during the seminar.

(4) Guest Lecture

Guest lecture was one of the highlights of the course. The lectures were very useful as they broaden our perspective. If possible, add a few more speakers to the next year's course.

(5) Teaching Assistants

Mike has done a superb job. He is a class of his own. Cheryl was diligent and very familiar with what she was doing. She was a great asset when we were doing our research. Margaret was a good T.A. She always showed keen interest in what we were doing. She was also very good in knocking off ideas with the students. Bobby was hardworking and very patient with the students.

(6) Syllabus

The syllabus was not completely covered as announced. Topics like nutrient analyses, gas fluxes, antibody preparation, fluorescence antibody detection were omitted.

(7) Text Book

The text book chosen was not quite relevant.

(8) Chemicals for Research

As long as the chemicals needed are not too expensive, they should be made easily available to the students eg. ligation buffer.

(9) Library

The library is impossible. If possible, press USC to upgrade it.

As this is the first time UCLA is offering the course, it is understandable that there are rooms for improvement. As a whole, the course is a success. I have learned a lot and my perspective has been broaden. Put it in another way, the course was a total re-education for many of us!

Congratulations and thank you again.

Tun-Liang Ong

Course Syllabus
April 3-14 Marine Molecular Segment

Monday April 3 - Students arrive

Evening Session - Overview of the molecular segment and relationship the of instruction to independent projects. Discussion of general laboratory safety; chemical, biological and radiation.

Tuesday April 4 -

Morning session - Radioisotope handling procedures and licensing of the class.

Laboratory instruction - Isolation and purification of DNA from sea urchins.

Wednesday April 5 -

Morning session - DNA isolation protocols for marine animals and plants, with emphasis on nuclease protection and tissue homogenization protocols.

Laboratory instruction - Characterization of isolated DNA by fluorometry and spectrometry. Determination of DNA size by field inversion electrophoresis.

Thursday April 6 -

Morning session - Principles of restriction analysis, Southern transfer to a hybridization matrix and in vitro labelling of DNA probes.

Laboratory instruction - Restriction analysis of isolated DNA, vacuum transfer of DNA to hybridization filters, in vitro labelling of probe DNA by oligonucleotide primed synthesis and probe hybridization protocols.

Friday April 7 -

Morning session - Cloning of recombinant DNA into plasmid vectors.

Laboratory instruction - Restriction of plasmid vectors for cloning, isolation of DNA fragments suitable for insertion into vectors, ligation of recombinant DNA molecules and preparation of transformation competent bacterial cells. Washing of hybridization filters and preparation of autoradiography.

Saturday April 8 -

Morning session - Considerations in selection of probes for population and evolutionary studies.

Laboratory instruction - Growth of minicultures from clones containing recombinant DNA, preparation and restriction analysis of DNA from recombinant clones.

Sunday April 9 - Open

Monday April 10 -

Morning session - Principles of genomic bank production, properties of lambda phage vectors and the formation of clone banks.

Laboratory instruction - Preparation of random genomic fragments suitable for insertion into lambda phage arms, characterization of the insert fragments by field inversion gel electrophoresis and the ligation of inserts with lambda arms.

Tuesday April 11 -

Morning session - Principles of the polymerase chain reaction (PCR), selection of appropriate primer sequences and general properties of this technique.

Laboratory instruction - In vitro packaging of recombinant lambda DNA, plating and titring of lambda genomic bank. Performance of PCR amplification on sea urchin mitochondrial and vitellogenin genes, characterization of PCR amplified DNA by gel electrophoresis and ligation into a plasmid.

Wednesday April 12 -

Morning session - Principles of DNA sequencing.

Laboratory instruction - Screening of lambda plaque libraries with labelled probes. Transformation of competent cells PCR recombinant DNA. Performance of the dideoxynucleotide sequencing reactions, preparation of sequencing gels, loading and electrophoresis of sequencing gels.

Thursday April 13 -

Morning session - Production and analysis of genomic libraries.

Laboratory instruction - Selection of PCR colonies and growth of minicultures. Selection of positive lambda plaques from screening, preparation of phage from individual plaques. Preparation of the sequencing gel for autoradiography.

Friday April 14 -

Morning session - Application of recombinant DNA techniques to marine ecological and evolutionary problems.

Laboratory instruction - Development and reading of DNA sequence autoradiogram. Characterization by restriction analysis of the PCR clones. Preparation and characterization of recombinant DNA from lambda phage.

WEEK 3

8:15 AM	LECTURES	1:15 PM	LABORATORY ACTIVITIES
MONDAY: April 17			
Intro to Cell Biol	Organelle separations: centrifugation, gradients, osmotica, etc.	Isolation of chloroplasts from <u>Codium</u>	
TUESDAY: April 18			
Metabolic compartmentation transport, permeability, temporal and spatial expression	Enzyme kinetics, isozymes, conventions	Sub-organellar fractionation enzyme and protein assays	
WEDNESDAY: April 19			
Membranes: composition, properties, enzymes	Protein purification: Salting out, molecular sieving, ion-exchange,	Fractionation of CARBONIC ANHYDRASE using FPLC	
THURSDAY: April 20			
Marine Algae I	Protein purification: reversed phase, affinity chromatography, etc.	continue	
FRIDAY: April 21			
Marine Algae II	Protein purification: continued.	Fractionation of membranes using HPLC	
SATURDAY: April 22			
*****	*****	continue	
SUNDAY: April 23			
	Round table discussion		

WEEK 4

MONDAY: April 24		
Guest lecture: Don Manahan	HPLC: small molecules	RNA isolation from <u>Codium</u>

8:15 AM	LECTURES	1:15 PM	LABORATORY ACTIVITIES
TUESDAY: April 25			
Biology and cell biology of symbiosis	Electrophoresis, protein blots	Purification of poly(A) ⁺ RNA, <u>in vitro</u> translation	
WEDNESDAY: April 26			
Biology and cell biology of marine symbiosis II	Immunotechnology	Immunoprecipitation, SDS-PAGE electrophoresis fluorography	
THURSDAY: April 27			
Cell biology of calcification		Lectin overlays, western blots, immunoblots	
FRIDAY: April 28			
Guest lecture: Linda Goff	Guest lecture	continue	
SATURDAY: April 29			
***		Fluorescence microscopy, dark room work	

MARINE MOLECULAR BIOLOGY

DEPARTMENT OF BIOLOGY UCLA
Graduate Training Course

Sponsored by the Office of Naval Research, DOD

Generous gifts and considerations are gratefully acknowledged from:

Pharmacia LKB Biotechnology, Inc.

Beckman Instruments, Inc.

Gilson Medical Electronics, Inc. and

Hoefer Scientific Instruments

The techniques and procedures in this manual have been developed or refined to serve the MMB course on Santa Catalina Is. Your comments and suggestions for improvement will be greatly appreciated. Have a great Spring.

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Unit 1.

Chloroplast Isolation and Purification of
Thylakoid Membranes of Codium fragile
Using Reversed Phase HPLC and SDS-PAGE

Introduction

This unit will introduce you to a strategy for rapid purification of proteins from a defined source. We have used this approach to good advantage for polypeptides from the glyoxysomal matrix. The purified polypeptides were used to generate monospecific antibodies and these, in turn, have been used as immunoprobes for screening a cDNA expression library.

As you will learn, there are a large variety of HPLC columns. The range of possible applications is, therefore, almost limitless. For our purposes, we have found reversed phase C_4 columns particularly useful. The columns can be used analytically, and have the great advantage of allowing you to survey and compare many samples in a short length of time. Once a sample is selected, it can be scaled to a semi-preparative mode and the complexity of the initial mixture in the sample can be reduced considerably. Because the hydrophobic properties of proteins are independent of their molecular mass, even moderately complex column fractions can be well separated on SDS PAGE (analytical or preparative gels). Thus, in tandem, the two techniques will permit isolation to homogeneity of a polypeptide that can be rigorously defined in terms of its affinity for the column in acetonitrile and its molecular mass.

Obviously, this approach is most useful when the knowledge of a protein's biological activity is not immediately the issue, but, rather, its location. For example, proteins from specific membrane fractions (ER, thylakoids, plasma membrane, etc.) can be isolated but their biological activity (enzymatic, receptor, etc.) will not be immediately known. However, if the protein is being purified for the purpose of generating antibodies to use in screening expression libraries, the eventually cloned and isolated DNA can be sequenced and the sequence obtained can be compared to known sequences in the sequence banks. By matching sequences, we have been able to verify the identity of 3 enzymes that we isolated from the matrix of the glyoxysomal matrix.

In this case study, we will try to isolate polypeptides from the thylakoids of Codium. It will be interesting to check our results on analytical gels and on immunoblots (western blots) using antibodies against 2 thylakoid polypeptides; the light harvesting complex protein (LHC) from diatoms and the Reaction Center P700 protein from spinach.

Chloroplast Isolation on Sephadex G-50

Steps Prior to Homogenization:

- * Place 100 ml of grinding medium in a Waring Blender container and put into a freezer 1 hours prior to homogenizing the plant.
 - * Resuspend 4 g Sephadex G-50 coarse in 60 ml Grinding Medium and let it sit overnight at 5°C.
 - * Store the fresh Codium in the cold room 1 day before use to de-starch the plant.
 - * All solutions must be 0-5°C and procedures must be carried out as rapidly as possible.
1. Use 10 gm of Codium and remove as much of the epiphytes as possible. Rinse with cold water and blot dry. Using a razor blade cut the plant into small (1 cm) pieces on a glass plate.
 2. Grind the mercerized tissue in a Warring blender, containing the ice chilled Grinding Medium, using up to three 5-second bursts with a 2-second interval in between.
 3. Filter the homogenate through four layers of cheese cloth placed on top of one layer of miracloth. Keep the filtrate on ice. Save 100 µl in eppendorf tube for protein and chlorophyll analysis (record the total volume).
 4. Centrifuge the filtrate at 1000x g for 7 minutes at 5°C.
 5. Resuspend the pellet in 50 ml grinding medium, gently using a camel hair brush. Centrifuge the resuspended pellet at 1000x g for 7 minutes at 5°C.
 6. Resuspend the pellet in 2 ml grinding medium gently using the brush.
 7. Prepare a loosely packed Sephadex G-50 Coarse Column (2 X 15 cm) in the cold room (prepare just before use).
 8. Gently layer the resuspended crude chloroplasts on the column. Collect the first 5 ml of the green band coming through the column. (At this step the chloroplast fractions of every person in one group can be combined together).
 9. Centrifuge the chloroplast fraction at 2000x g for 10 minutes at 5°C.
 10. Resuspend in 2 ml TE buffer + 0.6M sucrose. Allow to stand on ice for 10 minutes. Save 100 µl for protein and chlorophyll assay.
 11. Freeze this chloroplast fraction at -20°C for about 1.5 hr. Thaw at room temperature. Keep on ice when thawed.

12. Add 2 volumes of TE buffer to bring the sucrose concentration to 0.2M.
13. Homogenize this mixture in a glass homogenizer with 20 strokes to further rupture the chloroplasts.
14. Centrifuge the broken chloroplasts at 4500x g for 15 minutes at 5°C. Pellet is the thylakoid membranes. Discard the supernatant.
15. Dissolve the thylakoid membranes in 2-4 ml 0.5% Triton x-100R by vortexing (gently) (if the resuspended thylakoid membranes solution is flocculant you can add 1-2 ml more of the 0.5% Triton R.
16. Bring the concentration of Triton down to 0.05% by adding HPLC grade water. Save 100 µl for protein assay and another 100 µl for SDS-PAGE
17. Place 1.5 ml of the thylakoid membranes into Eppendorf tubes and centrifuge in microfuge for 5 minutes to prepare samples for HPLC analysis. Transfer the supernatants into a clean tube.

*note: Sample purification is an important step in using the HPLC. The aim is to remove extraneous materials in the sample in order to keep the column from plugging. This is accomplished just prior to injection by either of the two following methods: a) filtrating; or b) centrifugation.

Membrane Protein Purification by HPLC

HPLC Elution Program for Thylakoid Membranes

Solvents:

Pump A - 0.1% Trifluoroacetic Acid (TFA) in H₂O.

Pump B - 60% Acetonitrile in 0.1% TFA, H₂O.

Gradient - 0-100% B in 30 minutes.

Column - VYDAC C₄ Reverse Phase.

Flow Rate - 1.5 ml/min

Absorbance - 260 nm*

*205 or 215 nm or ~ 40 X more sensitive in detecting proteins (absorption by the peptide bond), however one must be careful because these wavelengths are also extremely sensitive in detecting impurities in the solvents. since you are using Triton X-100R, which has high absorbance at these wavelengths, this is not an option that you have. In a study of a different membrane, we are monitoring at 220 nm because it gives us a little better sensitivity than 260 nm.

Solvent preparation:

- a. Must always use HPLC grade solvents.
- b. All solutions must be filtered through a 0.5 μ m filter.
- c. All solutions must be degased with Helium, gently for about 15 minutes prior to use, in order to avoid production of air bubbles in pumps.

Sample preparation - just prior to injection using either of the methods below:

- a. Filtration through a 0.5 μ m filter
- b. Centrifugation

Note: To be on the safe side always clean the sample if it has been frozen and thawed or has been sitting around for several hours.

Column Care:

- a. Column is handled very gently.
- b. Column is connected paying attention to the direction of flow on the column.
- c. 2-3 column bed volumes of solvent is passed through the column prior to sample injection.
- d. The column must be washed with the proper solvent to elute any material which is attached to the column before storage.
- e. Column is stored in the proper solvent. The literature which comes with a column always indicates the proper storage solvent.

Final Care of the Instrument:

After washing the column in the storage solvent, it is removed and HPLC grade isopropanol or methanol is pumped through the instrument.

Chlorophyll Assay

1. Place 100 μ l of each suspension into separate eppendorf tubes. Add 800 μ l acetone and 100 μ l distilled H_2O , cap, vortex, and allow to stand on ice in dark for 5 minutes.
2. Centrifuge in a microfuge for 5 minutes, separate the supernatant by placing into clean eppendorf tubes. Keep the pellets on ice for protein determination.
3. Measure the chlorophyll in the supernatant by measuring the absorbance at A_{663} and A_{645} . Use 80% acetone for blank.

$$Chl (\mu g/ml) = [20.2 (A_{645}) + 8.02(A_{663})] \times \text{dilution factor (1000/100)}$$

Bradford Bio-Rad Protein Assay

Microassay Procedure:

1. Prepare dilutions of the Protein standard (BSA) from 1-25mg/ml.
2. Dissolve the protein pellet in 100 μ l 0.1N NaOH. Make appropriate dilutions of each sample using distilled H_2O and bring the volume up to 0.8 ml.
3. Place 0.8 ml of each Protein standard concentrations and the diluted samples into clean 13 mm X 100 mm test tubes. Place 0.8 and distilled H_2O in the blank tube.
4. Add 0.2 ml Dye Reagent, vortex.
5. After 5 minutes to one hour read the absorbance at 595 nm versus the blank.
6. Plot the absorbance of standard concentrations and determine the concentration of protein samples from the standard curve.

Since solubilization in detergent is affected by detergent-to-protein ratio, it is important that you know this ratio at all times. Refer to paper by Morrissey et al. (Eur. J. Biochem. 160:389-396).

Some Common Problems with HPLC

Symptom	Problem	Solution
Whinnying noise from the pumps.	Bubble in the pump.	Bubbles are removed by unscrewing the liquid head of the pump and allowing pumps to operate.
Erratic Pressure.	"	"
Steady high pressure.	Flow ratio is set too high. Column blocked.	Decrease flow rate. Backflush column. Replace column.
High pressure.	Air in pump.	De-gas solvent. Bleed air from pump.
Spikes in chromatogram or noisy base line.	Air in detector cell.	*Unscrew the detector panel (both screws at the same time). Inject HPLC grade methanol and then dry with helium gas and reassemble. Tighten the back pressure device.

*Please, don't undertake assembly or disassembly of HPLC components unless you have received specific instruction or are being supervised by TA or faculty.

Sample Preparation for SDS-PAGE

1. Each peak collected from the HPLC is freeze dried.
2. The freeze-dried fractions are dissolved in the smallest volume of sample buffer as possible (e.g. max 50 μ l).
3. The dissolved fractions are transferred into Eppendorf tubes and heated in a boiling water bath for 1 minute.
4. 10 μ l of each fraction is loaded onto the gel.
5. Analytical gels will be stained with silver.

For the preparation of the gel and buffers refer to the Hoefer Scientifics Instruction manual for Mighty Small II Slab Gel Electrophoresis unit.

Solutions and Materials

Solutions:

Grind Medium

9% Sucrose (w/v)	90 gm
0.2% BSA (w/v)	2 gm
50mM Tris-HCl (pH 7.8)	6.05 gm
adjust pH with HCl to 7.8	
adjust volume to 1 liter	

TE Buffer + 0.6% sucrose

10mM Tris-HCl (pH 8.0)	1.21 g Tris base
2mM EDTA	0.74 g Na ₂ EDTA.2H ₂ O
0.6% sucrose	6g sucrose
adjust pH to 8.0 with HCl	
adjust final volume to 1 liter	

TE Buffer

10mM Tris-HCl (pH 8.0)	1.21 g Tris Base
2mM EDTA	0.74g Na ₂ EDTA.2H ₂ O
adjust pH to 8.0 with HCl	
adjust final volume to 1 liter	

0.5% Triton 100-R

0.5 g Triton-x100-R
100 ml H₂O

80% (v/v) acetone

800ml acetone
200ml H₂O

0.1N NaOH

4 g NaOH
adjust volume to 1 liter

Sephadex G-50

Materials:

Materials:

Waring Blender

Miracloth

Cheesecloth

funnels

Camel Hair Brush

Centrifuge bottles (250 ml)

Centrifuge tubes (50 ml)

Columns (2 X 15 Cm)

Eppendorf Tubes

Glass Homogenizer

Vortex

Test Tubes 13 X 100

Razor blades

8 X 10 inch glass plate

stands & clamps for columns

Vydac C₄ column

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Unit 2.

Subcellular Localization and Purification of Carbonic
Anhydrase from Codium fragile

Introduction

Carbonic anhydrase catalyzes the hydration of carbon dioxide:



In animals, this metalloenzyme (Zn) catalyzes the reaction, as written, in the blood and muscles and the reverse reaction in the lungs. The rate of CA is one of the fastest known. It has a turnover number of 600,000/sec (compare to 15/sec for DNA polymerase I). [The turnover number is the number of substrate molecules converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated with substrate.] It hydrates CO_2 at 10^6 to 10^7 times the rate of the uncatalyzed reaction. Its K_m is 8 mM.

In marine algae the important reaction ought to be the reverse reaction. Since algae occupy habitats ranging from the intertidal (where they can spend part of their day fully emersed) to depths greater than 100 ft. they might be exposed to CO_2 (in air) or CO_2 and HCO_3^- in seawater, all in one day. However, regardless of depth, the pH of Seawater is 8.2 to 8.3, thus the expected form of dissolved inorganic carbon in the ocean is HCO_3^- . Since the substrate of ribulose biphosphate carboxylase/oxygenase (RUBISCO) is CO_2 , the CA in marine algae may well function as a mechanism for concentrating CO_2 provided that its subcellular compartmentation is appropriate.

In this case study we will examine some characteristics of the enzyme in Codium, and its subcellular localization. We will also use this enzyme to study some standard techniques for enzyme purification. Finally, we hope that our findings will provide clues concerning the regulation of the enzyme and, therefore, more insightful approaches that may lead to an understanding of its molecular regulation.

Subcellular localization: Summary

Our initial, plausible, hypothesis is that CA is localized in either cytosol or chloroplast (or both!). To address this hypothesis our approach might be as follows:

- Induce maximal levels of CA (0.1% sodium bicarbonate for 16 hrs).
- Determine total CA activity in a crude extract (make sure all of the organelles are broken), ascertain activity units per unit fresh weight.
- Isolate chloroplasts from a given weight of tissue, measure activity in the chloroplast sample, obtain activity in chloroplasts per unit fresh weight.
- Calculate percent of the activity in the chloroplasts.

In vitro Assay for CA Activity

Carbonic anhydrase activity is determined by measuring the decrease in pH after addition of substrate (CO_2). The decrease in pH is due to the production of H^+ and HCO_3^- .

1. Substrate preparation: A stream of CO_2 is passed through 1 liter of distilled water via an air stone for 10 minutes or until the pH reaches 3.5 or below. The CO_2 -saturated water is kept in a tightly stoppered-glass flask in the cold room or in an ice bucket.
2. 1 ml of the sample in 25 mM veronal buffer is placed into a small vial with a small stir bar and 1 ml of 50 mM veronal buffer, pH 8.3 is added. This mixture is stirred and the pH is monitored on a chart recorder.
3. 1 ml of CO_2 -saturated H_2O is added rapidly and the change in the pH is recorded.
4. The change of pH in 1 minute between pH 8 and 7 is determined.
5. Non-specific pH change is determined using 1 ml of the sample which is boiled for 5 minutes, and the change in pH is measured in one minute as described above.
6. Soluble protein is measured using the Bio-Rad micro assay procedure.
7. Carbonic anhydrase activity is then determined from:

$$\frac{(\Delta \text{ pH of native homogenate} - \Delta \text{ pH of denatured control}) \cdot \text{minute}^{-1}}{\text{mg soluble protein or fresh weight}}$$

Inhibition of CA activity:

Acetazolamide (Diamox) is a specific inhibitor of CA. It is very light sensitive, therefore, buffer containing Diamox should be kept away from light.

1. Inhibition is determined by substituting 50 mM veronal buffer containing 10^{-3} M Diamox for 50 mM veronal buffer in the in vitro assay.
2. Percent inhibition is calculated from:

$$100 - [\text{CA activity in presence of Diamox} / \text{CA activity}] \times 100$$

CA activity of the whole thallus:

1. 10 g of Codium fragile is rinsed with distilled water and ground in 20 ml of 25 mM veronal buffer.
2. The mixture is filtered through four layer of cheese cloth on top of one layer of miracloth.
3. The filtrate is frozen for 1.5 hrs, thawed, and placed in ice bath promptly. This ruptures the chloroplasts and releases soluble proteins.

4. The suspension is placed in a 30 ml glass homogenizer and further homogenized using 20 strokes in order to ensure chloroplast breakage.
5. Carbonic anhydrase activity of the whole cell is measured by the in vitro assay described earlier.

CA activity in chloroplasts:

1. 10 g of Codium is rinsed and ground in 20 ml of grinding medium. Intact chloroplasts are isolated as described in the previous experiment using a Sephadex G50 column.
2. Chloroplasts collected in the first 5 ml of green band from the column and centrifuged at 2000x g for 10 minutes, 5°C.
3. Chloroplasts in the pellet are resuspended in 10 ml of grinding medium and recentrifuged.
4. The pellet is resuspended in 5 ml of 25 mM veronal buffer and freeze/thawed and further ruptured by a glass homogenizer.
5. Carbonic anhydrase activity is determined by the in vitro assay.

Carbonic anhydrase activity of the whole cell is compared to the activity of the chloroplasts to determine localization of the activity.

This method of chloroplast isolation may not be adequate. If the enzyme is located in chloroplasts, it will be important to establish an accurate balance sheet for the activity in all the cell compartments. For example, if the enzyme is in the chloroplast - is it all in the chloroplast? One way to find out might be to spin out intact chloroplasts from a carefully lyzed-cell preparation. Then assay the Chl-free supernatant. If the supernatant has no activity then all the activity must be in the chloroplasts, etc.

Solutions and Materials

- 1) 25 mM Veronal Buffer:
25 mM Sodium Barbital 5.14 g
5 mM EDTA 1.86 g
5 mM DTT 0.77 g
10 mM MgSO₄ 1.69 g
0.1% BSA 1.00 g
adjust pH to 8.2 with NaOH
adjust volume to 1 liter distilled H₂O
- 2) 50 mM Veronal Buffer:
50 mM Sodium Barbital 10.28 g
5 mM EDTA 1.86 g
5 mM DTT 0.77 g
10 mM MgSO₄ 1.69 g
adjust pH to 8.2 with NaOH
adjust volume to 1 liter distilled H₂O
- 3) Grinding Medium:
9% sucrose (W/V) 90 g
0.2% BSA (W/V) 2 g
50 mM Tris-HCl 6.05 g
adjust pH to 7.8 with HCl
adjust volume to 1 liter distilled H₂O
- 4) 50 mM Veronal Buffer + 10⁻³ M Diamox
50 mM Sodium Barbital 10.28 g
5 mM EDTA 1.86 g
5 mM DTT 0.77 g
10 mM MgSO₄ 1.69 g
10⁻³M Diamox 0.22 g
adjust pH to 8.2 with NaOH
adjust volume to 1 liter distilled H₂O
- 5) Ammonium Sulfate
- 6) Sephadex G-50
- 7) CO₂ - Saturated H₂O

Materials:

Waring blender	pH meter and recorder
Razor blades	Scintillation vials
Glass plates	Small stir rods
Funnels	pH standards (4,7,10)
Cheese cloth	Eppendorf tubes
Camel hair brush	Glass homogenizer
Centrifuge tubes (50 ml)	Vortex
Columns (2 x 15 cm)	Test tubes (13 x 100)

References:

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Unit 3.
Isolation of Total RNA
From Codium fragile

Introduction

The ability to isolate clean, intact and biologically active RNA is an important tool in the field of molecular biology. For several applications, such as the production of a cDNA library, the use of full-length RNA is critical. This need has led to the development of several methods of RNA isolation (Ausubel, et. al., 1988). These methods vary in complexity and speed, and are dictated in part by the type of tissue under study. For example, plant tissues require different conditions for RNA isolation than do tissue culture cells. Algae possess a battery of problems unique to themselves (Su and Gibor 1988). The problem common to all the varying methods, however, is the presence of cellular ribonucleases. Ribonucleases are very stable, active enzymes that function without cofactors to degrade RNA molecules. The key to obtaining good preparations of RNA, then, is to minimize ribonuclease activity during the initial stages of the extraction, and to avoid the accidental introduction of RNases from contaminated glassware and solutions.

Our goal for the next few days is to isolate total RNA from Codium fragile, further purify the messenger RNA, and translate the mRNA in an in vitro wheat germ lysate translation system. The translation products will be examined by SDS PAGE and fluorography, and finally immunoprecipitated with an anti-Codium fragile lectin antibody.

The method we've chosen for RNA isolation (Logemann, et al., 1987) was designed to disrupt cells and inactivate ribonucleases simultaneously. The method uses the powerful chaotropic agent guanidine hydrochloride, which readily denatures and deactivates ribonucleases and rapidly disintegrates cellular structures. The nucleic acids are then separated from the dissolved cellular components by phenol extraction. Finally, an ethanol precipitation step separates the RNA from the DNA and residual proteins.

The total cellular RNA population obtained is roughly 80-85% ribosomal. 10-15% consists of low molecular weight species such as transfer RNAs and small nuclear RNAs. Generally, 0.5% to 5% of the total RNA is mRNA. Virtually all eucaryotic mRNAs carry a poly(A) tail on their 3' ends that allows the purification of mRNA by affinity chromatography on oligo dT cellulose columns or messenger affinity paper. The population of mRNAs obtained collectively encodes for all the polypeptides synthesized by the cell at the time of isolation.

One of the chief causes of failure during an RNA isolation procedure is the accidental introduction of ribonucleases from contaminated glassware and solutions. Given the stability of RNases, a small amount goes a long way. Fortunately, several precautions are available. Use them religiously.

1. Plasticware

Sterile, disposable plasticware is essentially RNase free and can be used without pretreatment. It is preferable to use as much plasticware as possible, including disposable pipets, centrifuge tubes, Eppendorf tubes, and Pipetman tips. Plasticware that is not sterile requires autoclaving for 20 minutes, particularly Pipetman tips (supplied in autoclavable boxes) and Eppendorf tubes. Non-autoclavable plasticware may be treated in 1.0% SDS at 70°C for 3 hours, and then rinsed thoroughly in DEPC-treated water (see below).

2. Glassware

All glassware used is a potential source of RNase contamination and must be baked at 250°C for a minimum of 4 hours. This includes all glassware used not only in the extraction of the RNA, but also that used to prepare solutions, such as beakers, graduated cylinders, storage bottles, flasks and so on. Cover all the openings with aluminum foil before baking.

3. Miscellaneous items

All other things used in the preparation of solutions or the RNA, such as spatulas, stirring bars, mortar and pestles, homogenizer parts, Pasteur pipets, etc., should be wrapped in foil and baked at 250°C for 4 or more hours.

4. Cuvettes

Quartz cuvettes are required to measure and quantitate the RNA produced. These cuvettes are expensive (\$75 - \$100 apiece). Treat them in 1.0% SDS at 70°C for 3 hours. Rinse thoroughly with DEPC-treated water.

5. Solutions

All solutions should be prepared in baked glassware using sterile water, and then stored in baked bottles. Ideally, the dry chemicals used should be reserved for RNA work and handled with baked spatulas.

Whenever possible, all water and salt solutions must be treated with the chemical diethylpyrocarbonate (DEPC) which inactivates RNases by covalent modification. Add DEPC to all solutions to a final concentration of 0.05% to 0.1%, and mix thoroughly. Allow the solutions to sit overnight at room temperature, then autoclave for 20 minutes. Please note that DEPC cannot be used to treat solutions containing Tris. DEPC is highly unstable in the presence of Tris buffers and decomposes to ethanol and carbon dioxide. Finally, DEPC is a suspected carcinogen. Handle it accordingly. Work in a fume hood and wear gloves.

6. Hands

Your hands are a major source of RNases. **ALWAYS WEAR GLOVES!**

7. General considerations

One cannot be too careful in preventing RNase contamination. It is a good idea to set aside and clearly mark all glassware and plasticware used for RNA preparation and reserve those items for RNA work only. Always handle these things with gloved hands. Use common sense, and be careful.

Materials

Virtis homogenizer, cups and blade assembly
Centrifuge, JA-0 rotor and adapters
30 ml corex tubes (6)
Oakridge tubes (12)
50 ml graduated cylinders (6)
15 ml corex tubes
RNase free cuvettes
Spectrophotometer
RNase free pipetman tips
Baked Pasteur pipets
Autoclaved Eppendorf tubes

Solutions:

1. Extraction buffer

8.0 M guanidine HCl	152.84 g
20 mM MES	0.85 g
20 mM EDTA	3.72 g
50 mM 2-mercaptoethanol	0.87 ml

Adjust pH to 7.0 before adding the 2-mercaptoethanol. Bring to 200 ml with distilled water.

2. Phenol/Chloroform/Isoamyl alcohol

25:24:1 (V:V:V)

Prepare 200 ml

Phenol (md. bio. grade): 100 ml : 100 ml

Chloroform : 96 ml

Isoamyl alcohol : 4 ml

Equilibrate twice with 2.0 M Tris, pH 8.0. Add an equal volume of the Tris solution, mix well, and allow the phases to separate (a separatory funnel works well here). Remove the lower organic phase, and extract it again with 2.0 M Tris, pH 8.0. Equilibrate the organic phase a final time with 0.1 mM Tris, pH 8.0. Remove the final organic phase and store frozen.

The phenol/chloroform/IAA solution may become cloudy during its preparation. Don't panic. The cloudiness disappears upon standing.

3. 3.0 M Na Acetate, pH 6.0 : 100 ml

NaOAc 24.6 g

Add 75 ml of distilled water and dissolve. Adjust pH to 6.0 with acetic acid, then bring to 100 ml final volume. Treat with 0.05% DEPC and autoclave overnight.

10M
4. ~~0.1 M~~ Acetic acid : 100 ml

Glacial acetic acid is 17.4 M. Dilute 5.75 mL of acetic acid to 100 ml with DEPC treated water. Use caution.

5. DEPC treated water

To 500 ml of water, add 250 μ l of DEPC. Mix well, and allow to stand overnight. Autoclave for 20 minutes.

Procedure

Pre-cool JA-0 rotor to 4°C.

1. Obtain 20 grams of fresh Codium fragile. Rinse briefly in fresh water. Remove epiphytic sections.
2. Freeze tissue as cold as possible. (At least -70°C; dry ice; preferably in liquid nitrogen).
3. Grind tissue to a powder in the Virtis homogenizer on high for 30 - 45 secs.
4. Add 40 ml GuHCl extraction buffer (Use 2 ml buffer per gram tissue). Grind at 50% speed for 2 min.
5. Transfer homogenate to baked 30 ml Corex tubes. Spin 10K rpm x 10 min at 4°C.
6. Carefully remove supernatant to a baked graduated cylinder. Divide supernatant into two autoclaved Oakridge tubes.
7. Add an equal volume of phenol/chloroform/isoamyl alcohol to each tube. Mix by hand inversion for 4 min.
8. Spin 10K rpm x 45 min at RT to separate phases.
9. Remove upper aqueous phase to a fresh, baked graduated cylinder. Discard the lower, brownish/green phenol layer. Place the phenol into the specified waste container.
10. Split the aqueous phase into two equal portions. Use Oakridge tubes. Add 0.2 vol. of 1.0 M acetic acid and 0.7 vol. of ethanol.
11. Store for 1 hr at -70°C.
12. Spin down the RNA 10K rpm x 10 min at 4°C. (White pellet forms).
13. Wash pellet 2 x with sterile, DEPC treated 3.0 M NaOAc, pH 6.0 at RT. Combine pellets into a 15 ml corex tube. Use 5 - 10 ml per wash. Spin RNA 10K rpm x 5 min after each wash.
14. Wash pellets in ¹⁰15 ml 70% ethanol. Spin at 10K rpm x 5 min.

Remove rotor and allow to warm as much as possible

Everybody spins together to save time

This step precipitates the RNA, leaving the DNA and residual proteins behind in the supernatant.

May go overnight at -20°C

The pellets won't dissolve, but become fluffy. Carefully resuspend them with a Pasteur pipet or pipetman.

15. Remove residual ethanol under vacuum (Lyophilizer is best. Speed vac. okay). Place tubes under vacuum for 2-3 min.

16. Redissolve pellets in DEPC treated water. Use about 2.0 ml. Be patient. Allow time for the RNA to go back into solution. Gently mix by hand.

Use RNase free cuvettes

17. Dilute 4 μ l of the RNA in 996 μ l of DEPC treated water (1000 μ l final vol.) in a RNase free cuvette. Measure the OD at 230, 260 and 280 nm. The concentration of RNA in μ g/ μ l is 10 times the OD₂₆₀ reading, assuming that 1 OD unit equals 40 μ g RNA per ml. Expect a yield of about 3 mg of total RNA from 20 g of Codium fragile.

The ratio of the OD₂₆₀ to the OD₂₈₀ readings is a measure of RNA purity. If the ratio is about 2, the sample is reasonably clean. If it's below 1.6, the absorbance is more likely due to proteins or some U.V. absorbing contaminant. Repeated ethanol precipitations are indicated, although this depends on the intended use of the RNA.

Polyphenol contamination is estimated by the ratio of the OD₂₃₀ to the OD₂₆₀. Polyphenols are a component of macroalgae that bind to macromolecules by hydrogen bonding or by covalent bonding when quinones form upon oxidation of the polyphenols. They are potentially strong inhibitors of RNA activities. A ratio value below 0.45 is considered safe.

18. RNA is best stored as an ethanol precipitate at -20°C or lower, although short term storage of the resuspended RNA at -20°C or -70°C is acceptable.

Ethanol precipitate the RNA by adding 1/10 volume of 3.0 M NaOAc, pH 6.0 and two volumes of cold ethanol. Mix well, and place the solution in the freezer. The precipitated RNA may be recovered by centrifugation (10K rpm x 10 min.) after one hour.

19. Proceed with the Poly(A)⁺ RNA isolation procedure using the resuspended RNA from step (16).

References:

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Unit 4.

Isolation and Purification of Poly(A)⁺
mRNA using Hybond_{TM} Messenger Affinity Paper

Introduction

Hybond-mAP is a specially derivatized paper that permits the simple and rapid purification of poly(A)⁺ RNA. Hybond-mAP is produced by covalently binding poly(U) chains to diazo-thiophenyl paper. Since most eucaryotic mRNA molecules possess a 3'-poly(A) tail, they bind specifically to the Hybond-mAP poly(U) chains via hydrogen bonding. This allows for the removal of the poly(A)⁻ RNA from a total cellular RNA population by a series of salt washes, leaving the poly (A)⁺ RNA behind. After a 70% ethanol wash to remove residual salt, biologically active mRNA is released by heating the Hybond-mAP/RNA complex in distilled water to disrupt the hydrogen bonds. The poly(A)⁺ RNA thus obtained is suitable for translation in an in vitro translation system, Southern blotting, and other analytical procedures.

The traditional method of poly(A)⁺ RNA purification involves the use of oligo dT cellulose chromatography (Aviv and Leder, 1972). Contaminants present in total cellular RNA populations obtained from macroalgae, however, bind to the cellulose used in the columns. These contaminants elute with the poly(A)⁺ RNA and inhibit translation, especially for red and brown algae. So far, our experience with mAP has avoided this problem. In fact, our initial attempts to translate Codium fragile mRNA purified on oligo dT cellulose failed.

Continue to exercise caution here regarding RNase contamination. Use RNase-free glass and plasticware, and always wear gloves.

Materials

15 ml Corning orange cap tubes
Baked scissors and forceps
mAP paper
Baked 100 ml beakers (6)
Shaker table
Ice
Sterile filter paper
Autoclaved Eppendorf tubes
Microfuge
Spectrophotometer
RNase-free cuvettes

Solutions

- 1) 5.0 M NaCl: 100 ml
NaCl: 29.22 g
H₂O to 100 ml
- 2) 0.5 M NaCl: 500 ml (make 2 batches)
NaCl: 14.61 g
H₂O to 500 ml
- 3) 0.15 M NaCl: 500 ml
NaCl: 4.38 g
H₂O to 500 ml

Add DEPC to 0.1%. Mix well. Stand overnight, then autoclave 20 minutes.

- 4) 70% Ethanol
100% Ethanol: 187.50 ml
DEPC • H₂O to 250 ml
- 5) DEPC treated water
Add 50 µl of DEPC to 100 ml of water. Mix and allow to stand overnight. Autoclave for 20 minutes.

Procedure

Set water bath to 65°C

1. Remove 1.0 mg of total RNA and add DEPC treated H₂O to 1.8 ml final vol. Place RNA in 15 ml Corning orange cap culture tube.

2. Using gloves and a baked pair of scissors, cut a 1x4 cm piece of Hybond mAP (stored at -20°C). Use 1 cm² of mAP per 0.25 mg total RNA.

3. Place the mAP into 20 ml (5 ml/cm²) of 0.5 M NaCl in a baked 50 or 100 ml beaker. Gently shake at room temp for 5 minutes.

4. Heat the RNA from step (1) at 65°C for 5 min to denature the secondary structure. Immediately cool the RNA on ice.

Re-set water bath temp to 70°C

5. Add 200 µl of 5.0 M NaCl to make 0.5 M NaCl in 2.0 ml final volume.

6. Transfer the Hybond-mAP to the cooled RNA solution. Lay the tube on a shaker table so that the RNA covers the mAP. Shake gently at room temperature for 45 minutes.

7. Transfer the mAP to a sterile 100 ml beaker. Wash the paper as indicated below, agitating the beakers by hand.

- A) 0.5 M NaCl - 40 ml. 5 min at room temp.
- B) Repeat (A).
- C) 0.15 M NaCl - 40 ml. 5 min at room temp.
- D) 70% ethanol - 50 ml. 3 min at room temp with shaking.

Sterilize filter paper:
Wrap in foil and autoclave

8. Remove the mAP from the ethanol. Allow to air dry on sterile filter paper for about 10 min.

9. Cut the mAP crosswise into 1/8 inch long sections using ethanol washed dissection scissors. Place the sections into an autoclaved Eppendorf tube.

10. Add 0.5 ml of DEPC treated H₂O to the tube. Make sure the mAP sections are covered. Incubate at 70°C for 5 min.

11. Transfer the aqueous phase to a fresh Eppendorf tube. Spin the remaining mAP paper in the microfuge for 30 sec. Transfer the residual liquid to the Eppendorf tube.

12. Measure the OD₂₆₀ and OD₂₈₀ of the entire sample. Use RNase-free cuvettes. Determine the OD₂₆₀ to OD₂₈₀ ratio. The total amount (µg) of RNA is determined with the following equation:

$$\text{OD}_{260} \times \text{Vol (in ml)} \times 40 \frac{\mu\text{g}}{\text{ml}} = \# \mu\text{g}$$

13. Proceed with the in vitro translation of the mRNA using the wheat germ lysate in vitro translation kit.

14. The remaining mRNA may be stored frozen (-70°C) as an aqueous solution. The best way to store RNA, however, is as an ethanol precipitate. Add 1/10 volume 3.0 M NaOAc, pH 6.0 and 2 volume ice-cold ethanol. Mix. Store at -70°C.

References

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Unit 5.

In Vitro Translation of
Codium fragile mRNA

Introduction

Having purified mRNA from Codium, our next task is to translate in vitro the purified messenger RNA into protein in the presence of a radioactively labelled amino acid, using a commercial in vitro translation system. Commercial translation systems have been prepared from such sources as rabbit reticulocytes, wheat germ and yeast. The wheat germ system offers some advantages for translating mRNA from plant sources, and therefore was the system selected for our purposes.

The wheat germ lysate has been fractionated to remove the endogenous amino acids and to reduce the potassium ion concentration. By adding potassium back to the lysate in varying amounts, the translation of an added, specific mRNA may be optimized. The lysate was also carefully treated to remove endogenous mRNA. The removal of this mRNA reduces background incorporation and contamination of the newly translated proteins by wheat germ proteins.

³⁵S The removal of the amino acids allows the system to use a variety of radioactively labelled amino acids. The available radio-nuclides include ²⁵S, ³H, ¹⁴C and ⁷⁵Se. We will use ³⁵S-methionine, and add on unlabelled mixture of the remaining 19 amino acids. Briefly, ³⁵S emits beta particles; the beta particles travel about 24 cm. in air, and will register on a geiger counter. The half-life of ³⁵S-methionine is 87.4 days. MilliCurie quantities of ³⁵S do not present a significant external exposure hazard because the low energy emissions barely penetrate the horny outer layer of skin. However, ³⁵S will volatilize and consequently spreads quite readily. Exercise the following precautions.

1. Wear gloves and a lab coat. Change gloves periodically.
2. Open ³⁵S-methionine storage containers under a fume hood if possible.
3. Do not smoke, eat or drink in an area where ³⁵S is used.
4. Use transfer pipets, spill trays and absorbent coverings to confine contamination.
5. Using a geiger counter, regularly monitor and promptly decontaminate gloves and work surfaces.
6. Isolate, label and dispose of waste following the approved guidelines. Your T.A. will instruct you where and how to dispose of liquid and dry wastes.
7. On completing an operation, secure all ³⁵S, remove and dispose of gloves, monitor and decontaminate self and surfaces.
8. Wash your hands.

The wheat germ lysate itself is sensitive to warm temperatures. The lysate has been divided into 50 µl aliquots and stored at -70°C. Use only what is necessary. Keep thawed lysate on ice and re-freeze as quickly as possible.

Continue to exercise caution regarding RNase contamination during the preparation of the translation reactions. Wear gloves and use RNase free-supplies. Once the translations have been completed, the threat of RNase contamination is no longer a problem. Be aware of the radioactivity, and continue your cautious ways. Don't contaminate yourself, the lab, or your T.A.

Successful translations yield a mixture of free ^{35}S -methionine and labelled proteins. The two must be separated to determine the effectiveness of the translation. A small aliquot (1-2 μl) from each translation is spotted onto a filter paper square. The proteins are precipitated onto the paper with trichloroacetic acid (TCA), and the unincorporated label washed off in warm TCA. After drying, the filters are then counted in a scintillation counter, and the total number of TCA precipitable counts per minute (cpm) determined. Cpm's approaching 1×10^6 and above are desirable, although 3 or 4×10^5 cpm are sufficient for our purposes.

Finally, we will examine the labelled proteins by SDS PAGE and fluorography after immunoprecipitation of the translation products with an anti-Codium fragile lectin antibody.

Materials:

Wheat germ translation kit (Amersham)	Scintillation fluid
^{35}S -methionine (50 μl aliquots)	Forceps
Filter paper squares	Styrofoam block with pins
100 ml beakers	Geiger counter
<u>Codium fragile</u> mRNA	Radioactive waste containers
LS 1801 Scintillation counter	Sterile Eppendorf tubes
Scintillation vials	

Solutions:

100% W/V Trichloroacetic acid (TCA)

1. Add 175 ml of distilled water to a 500 g bottle of TCA. Warm in a 37°C water bath until the TCA dissolves.
2. Pour the solution into a graduated cylinder. Adjust the volume to 500 ml with distilled H_2O .
3. Return the TCA solution to the original bottle. Store at 4°C .
4. Dilute as required.

Procedure: (See translation kit instruction booklet.)

Pellets are not always visible.
Be careful.

Set three water baths to the following temperatures: 25, 37, and 65°C. If the room temperature is greater than 25°C, then use ambient conditions.

1. Begin with mRNA from step (13) of the poly (A)⁺ RNA isolation procedure. If the RNA has been ethanol precipitated, spin it down at 10K rpm X 10 min. Remove the residual ethanol.

2. Remove an aliquot of the resuspended mRNA and adjust the mRNA concentration to 0.2 µg/µl with DEPC treated water. Alternatively, resuspend the ethanol precipitated mRNA to about 0.2 µg/µl. Allow time for the precipitated mRNA to dissolve.

3. Ideally, the translation kit requires optimization by adjusting the K acetate and mRNA concentrations in the reaction mixture. Because our immediate goal is to see if we have isolated translatable mRNA, we will use K acetate and mRNA at these initial amounts.

K acetate: 3 µl per reaction
mRNA : 0.2 or 0.6 µg per reaction

Additionally, the amount of ³⁵S-methionine used should fall between 15 to 30 µCi per reaction. (These reagents may be optimized later following the instructions supplied with the kit).

4. Set up four translations

#	Sample
1	Codium 0.2 µg mRNA
2	Codium 0.6 µg mRNA
3	Globin mRNA 0.1 µg
4	Background - no mRNA (H ₂ O)

Globin mRNA is used as a positive control.

Translation #3 is a positive control.
Translation #4 is a negative control, permitting the examination of the incorporation caused by endogenous mRNA.

5. When performing multiple translations, make up a large premix of reagents and then sub-divide that into the reaction tubes. Make up slightly more premix than required to allow for pipetting errors.

Pre-mix components:

Reagent	$\mu\text{l}/\text{trans.}$	# of trans.	Total
Amino acids	2.0	X	4.5 = 9.0
KOAc	3.0	X	4.5 = 13.5
Wheat germ	<u>15.0</u>	X	4.5 = <u>67.5</u>
	20.0		90.0

To prepare mix:

A) Remove KOAc, amino acid mixture and ^{35}S -Met solutions from storage, thaw, and mix thoroughly. Store on ice.

B) Just before use, remove the globin mRNA solution and wheat germ lysate from storage and thaw by hand heat. Mix gently by inversion, and store on ice. The extract must be stored on ice for the shortest time possible. Re-freeze immediately.

C) Combine the above into one sterile Eppendorf tube. Mix gently but thoroughly.

6. Pipet the following - in order - into sterile, autoclaved Eppendorf tubes kept on ice. Each tube should have 30 μl final volume.

This is an example. The amount of ^{35}S -Met required is estimated. The actual amount necessary will be determined based on the specific activity of the ^{35}S on the day it's used. See the decay table.

Solution	Translation			
	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
DEPC•H ₂ O	6.0	6.0	6.0	7.0
mRNA	1.0	2.0	--	--
Globin	--	--	1.0	--
Mix	20.0	20.0	20.0	20.0
^{35}S -Met	3.0	3.0	3.0	3.0

Work quickly!

7. Mix by gentle agitation. Briefly spin the reactions to return the solutions to the bottom of the tube.

Warm 10% TCA to 65°C.

8. Incubate at 25°C for 1 hour.

9. Meanwhile, return all solutions to storage. Ethanol precipitate the mRNA.

Prepare 1 cm² pieces of filter paper (i.e. Whatman #1) and label 1-4. Pin the filter paper squares to a styrofoam block.

10. (Optional) Heat the tubes at 37°C for 10 min. to hydrolyze the aminoacyl-tRNA complexes.

Store the translations at -70°C .

Dispose of the 10% TCA as radioactive waste. Carefully and thoroughly rinse and wash all contaminated glassware.

11. TCA precipitate the proteins to separate incorporated label from unincorporated ^{35}S -Met.

- A) Spot 1.5 μl from each translation onto a filter paper square. Allow to dry.
- B) Place the filter papers together into a beaker of 10% TCA. Incubate at 65°C for 5 min.
- C) Transfer the papers to fresh 10% TCA and incubate as above.
- D) Transfer the papers to 100% ethanol at RT for 2 min.
- E) Air dry filters on filter paper.

12. Place each dried filter into a separate scintillation vial, add scintillation fluid, and count over a 0-1000 energy window using the Beckman LS 1801 Scintillation Counter.

13. Determine the total cpm for each sample. If the translation cpm's exceed the background (tube #4) cpm by at least a factor of 4, then translation has occurred.

14. Proceed with SDS PAGE and fluorography of the total translation products, and with the immunoprecipitations.

References:

Instruction booklet by Amersham

Roberts, B.E., and Paterson, B.M. 1983. Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. Proc. Nat. Acad. Sci. USA 70:2330-2334.

Unit 6.

Immunoprecipitation of Proteins
Translated In Vitro

Introduction

Immunoprecipitation is a very useful technique that allows the selective removal of a specific polypeptide from a complicated mixture of proteins. The isolated polypeptide, which is normally labelled either by in-vitro translation or in vivo, is suitable for analysis by SDS PAGE and fluorography. By comparing in vivo labelled, immunoprecipitated proteins to proteins immunoprecipitated from an in vitro translation system, one may observe evidence of post-translational processing of a given polypeptide. Alternatively, immunoprecipitation allows an examination of a protein obtained from different stages in a time course labelling experiment. Several other applications of immunoprecipitation exist; we will concentrate on immunoprecipitation from translation products.

Immunoprecipitation relies upon an antibody to the protein in question, and the affinity ligand Protein A-Sepharose CL-B. Protein A is a protein found in the bacteria Staphylococcus aureus, and binds specifically to IgG-type antibodies. Protein A-Sepharose CL-B is protein A covalently coupled to the cross-linked matrix Sepharose CL-B. The sepharose has the form of tiny beads, which permits the rapid removal of the protein A-Sepharose from a solution by centrifugation in a microfuge. Briefly, a small amount of antibody is added to an aliquot of the translation products and allowed to react with the antigen, forming antibody-antigen complexes. Next, the antibody-antigen complexes will bind to protein A-Sepharose added to the mixture. The sepharose beads are then removed by centrifugation, and washed to eliminate non-specifically bound proteins. Finally, the antibody-antigen-Protein A complexes are dissociated by boiling in an SDS PAGE sample buffer, and then electrophoresed on a acrylamide gel and visualized by fluorography.

The antibody we will use is an antibody raised in rabbits against the lectin from Codium fragile, subspecies tomentosoides. The lectin was obtained commercially (Sigma), and is a 60,000 dalton tetramer. The 15,000 dalton subunits are identical. The subunits are bound by disulfide bridges to form the native protein, which is easily dissociated into subunits with mercapto ethanol. The lectin has a binding specificity for n-acetyl-galactosamine.

We plan to evaluate two methods of immunoprecipitation. The first method (Roberts, 1981), is exactly as outlined above, where the antibody reacts with the antigen before the addition of Protein A-Sepharose. The second method (Guidice, 1979) uses Protein A-Sepharose previously saturated with the antibody, so that the antigens form complexes with antibodies already bound to Sepharose. Each method has its advantages and disadvantages. The method of Roberts requires very little antibody material (some antibodies are very precious), but sometimes has background problems caused by non-specifically adsorbed proteins. Guidice's method results in very clean gels (no background), yet requires quite a bit of antibody to saturate the Protein A-Sepharose. Protein A-Sepharose has a binding capacity of 20 mg IgG per ml of the swollen Protein A-Sepharose gel.

You will continue to work with ³⁵S-methionine, so exercise caution. Wear gloves, and monitor yourself and work area periodically for contamination. We will use the protease inhibitor phenylmethylsulfonylfluoride (PMSF). PMSF is highly toxic. Be careful. Immunoprecipitation is a straight-forward procedure, but you must pay attention to details.

Stock Solutions

1. 1.0 M Tris, pH 7.5

Tris: 6.05g
H₂O to 50 ml

Adjust pH to 7.5 with 1.0 N HCl

2. 0.5 M EDTA

EDTA: 9.31g
H₂O to 50 ml

Allow time for this to dissolve.

3. 5.0 M NaCl

NaCl: 14.61
H₂O to 50 ml

4. Phenylmethylsulfonylfluoride (PMSF)

Dissolve 20 mg PMSF in 1.0 ml 100% ethanol. PMSF is a protease inhibitor. On the day of use, add it to the amount of buffer needed.

Store the stock solution at 20°C.

5. 20% SDS

SDS: 10g
H₂O to 50 ml

Roberts immunoprecipitation solutions

1. Solution #1

1.0% NP-40
10 mM Tris, pH 7.5
2 mM EDTA
150 mM NaCl
40 µg/ml PMSF

NP-40: 1.0 ml
Tris : 1.0 ml
EDTA : 0.40 ml
NaCl : 3.0 ml
H₂O to 100 ml

NP-40 (Nonidet P-40) is a detergent. Add the PMSF from the ethanol stock solution to the required amount of buffer needed for a day's work.

2. Solution #2

0.2% NP-40
10 mM Tris, pH 7.5
2 mM EDTA
150 mM NaCl

NP-40: 0.2 ml
Tris : 1.0 ml
EDTA : 400 µl
NaCl : 3.0 ml
H₂O to 100 ml

3. Solution #3

0.2% NP-40
10 mM Tris, pH 7.5
2 mM EDTA
0.5 M NaCl

NP-40: 0.2 ml
Tris : 1.0 ml
EDTA : 400 µl
NaCl : 10 ml
H₂O to 100 ml

4. Solution #4

10 mM Tris, pH 7.5

Tris: 1.0 ml
H₂O to 100 ml

Guidice immunoprecipitation solutions

1. Pellet buffer

120 mM Tris, pH 7.5
1.0% (w/v) SDS

Tris: 120 μ l
SDS : 50 μ l
H₂O : 830 μ l

2. Wash buffer

50 mM Tris, pH 7.5
0.15 M NaCl
10 mM EDTA
1.0% (w/v) Triton X-100
40 μ g/ml PMSF

Tris: 2.5 ml
NaCl: 1.5 ml
EDTA: 1.0 ml
Triton X-100: 0.5 g
H₂O to 50 ml

Add the PMSF from the 20 mg/ml stock solution as required.

3. 20% (w/v) TCA

Dilute the 100% (w/v) stock as required.

4. Ether: Ethanol (2:1)

ether: 6 ml
ethanol: 3 ml

Use the fume hood if possible.

Preparation of Protein A-Sepharose CL-B

1. Weigh out 0.75g of Protein A-Sepharose powder. Place it into a 15 ml Corex tube.
2. Add 8.0 ml of Roberts solution #1.
3. Allow to swell at room temperature for 2 hours.
4. Spin down the Sepharose 3K rpm x 5 minutes.
5. Resuspend the pellet in 5.0 ml of 1.0 M Tris, pH 7.5. Let stand for 30 minutes.
6. Transfer the Sepharose to a 15 ml Corning orange cap tube. Spin down in the centrifuge 1000 rpm x 2 min.
7. Note the volume of the packed pellet. Carefully remove the supernatant with a Pasteur pipet.
8. Resuspend the Protein A-Sepharose in 5.0 ml of Roberts solution #1. Determine the ratio of packed volume to final volume and maintain this ratio by adjusting the buffer amount as required.
9. Add NaN_3 to 0.02%. Store at 4°C.

Preparation of antibody saturated Protein A-Sepharose CL-B.

1. Incubate the swollen protein A-Sepharose beads with an excess of antibody overnight at 4°C on an end-over-end shaker. Use at least 20 mg IgG per ml of swollen gel.
2. Wash extensively with Guidice wash buffer. Pellet beads, and resuspend with excess buffer several times.
3. Wash with tris buffered saline (10 mM tris, pH 7.5, 0.15 M NaCl).
4. Resuspend the packed beads in a equal volume of tris buffered saline.
5. Add NaN_3 to a final concentration of 0.02%. Store at 4°C.
6. Antibody saturated protein A-Sepharose is stable for at least 2 months.

Materials

Shaker table
Translation products
Protein A-Sepharose CL-B
Eppendorf tubes
SDS PAGE equipment and solutions

X-ray film
Intensifying screens
X-ray cassettes
Photographic chemicals
Normal rabbit serum
Antibodies

Roberts Immunoprecipitation Procedure

Remember to add PMSF to solution #1.

Discard the first tube as radioactive waste.

NRS-rabbit serum obtained from an animal before it was injected to make antibodies.

Agitate periodically by hand to keep the PAS suspended.

Discard the pellet as radioactive waste.

The antibody may be in the form of serum, or purified IgG dissolved in buffered saline.

The more agitation of the beads, the better. Keep them suspended in solution.

Discard all supernatants and wash solutions as radioactive waste.

The pellets may be stored at -20°C.

1. Remove 300,000 to 400,000 TCA precipitable cpm's from a translation reaction and place them into an Eppendorf tube.

2. Add 300 to 500 μ l of solution #1.

3. Let stand at room temperature for at least 30 minutes.

4. Spin for 3 minutes in the microfuge. Carefully remove the supernatant to a fresh Eppendorf tube.

5. To the supernatant, add 2.0 μ l of normal rabbit serum (NRS). Let stand at RT for 15 minutes.

6. Add 40 μ l protein A-Sepharose (PAS). Let stand with occasional shaking at RT for one hour.

7. Spin 1.5 minutes in the microfuge. Remove the supernatant to a fresh tube.

8. To the supernatant, add 2.0 to 7.0 μ l of antibody. Let stand for at least 30 minutes at room temperature.

9. Add 40 μ l of PAS. Let stand for at least 30 minutes with occasional shaking.

10. Spin down the PAS for 1.5 minutes in the microfuge. Discard the supernatant.

11. Wash the pellet 4 times with 0.75 ml of solution #2 for each wash. Vortex the pellet in the solution for 30 seconds, then spin it down for 1 minute in the microfuge for each wash.

12. Wash as above with solution #3 two times.

13. Wash once with solution #4.

14. Spin down and save the pellet.

15. Vortex the pellet into 30 μ l of SDS PAGE sample buffer.

16. Heat in a boiling water bath for 2 minutes.

17. Spin down the beads in the microfuge for 2 minutes.

Remember to run 1.0 μ l of ^{14}C -methylated molecular weight standards also.

Discard the beads as radioactive waste.

18. Remove the supernatant and load it onto a previously prepared 10% acrylamide, 0.1% SDS gel. Use a Hamilton syringe.

19. Run the gel to completion and process for fluorography.

Guidice Immunoprecipitation Procedure

Preheat water baths to 37°C and 70°C.

Prepare 5 ml of 20% TCA and chill on ice.

The TCA precipitates the protein, leaving the unincorporated ^{35}S -methionine in the supernatant.

Dispose of all supernatants as radioactive waste.

Prepare 10 ml of ether: ethanol (2:1) and chill on ice.

Wash the pellets well.

Residual TCA can cause several problems.

1. Remove 300,000 to 400,000 TCA precipitable cpm's of translation products from a selected reaction.

2. Adjust volume to 25 μ l with H_2O .

3. Add 25 μ l of ice cold 20% TCA.

4. Incubate on ice for 20 minutes.

5. Spin in the microfuge for 2 minutes. Remove the supernatant.

6. Wash the pellet in ether ethanol (2:1): vortex, spin 2 minutes and remove the supernatant. Repeat 2 more times.

7. Remove the residual ether: ethanol by evaporation in a fume hood. You can accelerate the process by carefully blowing air or nitrogen over the pellet.

8. Add 50 μ l of pellet buffer to the pellet. Incubate at 37°C for 30 minutes to solubilize the proteins.

9. Transfer the samples to the 70°C bath and incubate 10 min.

10. Cool to room temperature.

Remember to add PMSF to the wash buffer.

We may have to improvise something here.

Discard washes as radioactive waste.

11. Dilute with 450 μ l of wash buffer.

12. Let sit at RT for 10 minutes. Spin in the microfuge for 1 minute, then transfer the supernatant to a fresh tube.

13. Add 50 μ l of the 1:1 suspension of anti-Codium lectin saturated protein A-Sepharose in wash buffer.

14. Agitate overnight at 4°C. An end over end shaker works well.

15. Next day: Spin down the protein A-Sepharose in the microfuge for one minute.

16. Remove the supernatant. Wash four times with 0.5 ml of wash buffer each time.

17. Wash the pellet once with 0.5 ml 10 mM Tris, pH 7.5 (Roberts solution #4).

18. Resuspend (vortex) the final pellet in 30 μ l of SDS PAGE sample buffer.

19. Heat in a boiling water bath for two minutes.

20. Spin down the beads for one minute in the microfuge.

21. Using a Hamilton syringe, carefully load a 10% acrylamide, 0.1% SDS gel. Also load 14 C-molecular weight markers.

22. Run the gel to completion and prepare for fluorography using sodium salicylate.

References

- Guidice, L.C., Waxdal, M.J. and Weintraub, B.D. 1979. Comparison of bovine and mouse pituitary glycoprotein pre- α subunits synthesized in vitro. Proc. Nat. Acad. Sci. USA 76:4798-4802.
- Roberts, L.M., and Lord, J.M. 1981. Synthesis and post translational segregation of glyoxysomal isocitrate lyase from castor bean endosperm. Eur. J. Biochem 119:43-49.
- Rogers, D.J., Loveless, R.W., and Balding, P. (1986) Isolation and characterization of the lectins from sub-species of Codium fragile. Lectins, Biology, Biochemistry, Clinical Biochemistry, Eds. Bog-Hansen, T.C., van Driessche, 5:155-160.

Immunoprecipitation from wheat germ in vitro translation

L. C. Guldice (1979) Proc Natl Acad Sci USA 76, 4798-4802

7. 25 μ l in vitro translation
8. 25 μ l 20 % ice cold TCA

incubate 20 min on ice

Spin in microfuge 2 min. Aspirate off TCA with drawn out pasteur pipet
Rinse pellet with ether:EtOH (2:1), spin, aspirate off supernatant, rinse
same way again, and let evaporate ether (work in the hood, dispose of
radioactive waste properly!)

Resuspend pellet in 50 μ l pellet buffer (or 25 μ l sample buffer when you
want to run all on SDS-PAGE)

pellet buffer:

830 μ l H₂O
50 μ l 20 % SDS
120 μ l 1 M Tris

incubate at 37 C until precipitate is solubilized (\approx 30 min).

incubate 10 min at 70 C, let cool to RT.

Dilute with 450 μ l wash buffer

wash buffer

43.5 ml H₂O
2.5 ml 1 M Tris (7.5)
1.5 ml 5 M NaCl
1 ml 0.5 M EDTA
0.5 ml Triton X-100
1 ml Aprotinin (Sigma)

→ on the day of use add protease inhibitor to the amount of buffer you will
need.

incubate 1 hr at 4 C, spin in Eppendorff, transfer supernatant to fresh tube,
add 50 μ l suspension of antibody saturated protein-A Sepharose
beads. In competition experiments add "cold" antigen before adding the
beads.

incubate ON at 4 C on an end over end shaker.

spin 10 sec in microfuge, remove supernatant with drawn out pasteur pipet,
wash four times with 0.5 ml wash buffer

To the washed pellet add 25 μ l sample buffer, mix well.

sample buffer:

250 μ l H₂O
100 μ l 1 M Tris
600 μ l 60 % sucrose
50 μ l 20 % SDS

add 5 μ l 1 M DTT, incubate 10 min 70 C.

add 5 μ l iodoacetamide (90 mg/ml), incubate 30 min 37 C.

Run samples on SDS-PAGE.

Notes:

The TCA precipitation gets rid of the vast amount of free radioactive amino acid, you will have low background on SDS-PAGE.

To saturate protein-A Sepharose with antibody, incubate beads with excess amount of antibody ON at 4 C on end over end shaker. Wash extensively with wash buffer (on sintered glass filter), then with TBS, add NaN₃ to a final concentration of 0.02 % and store at 4 C (stable for at least 2 months). Before use carefully resuspend the beads (shake end over end).

Unit 7.

Sodium dodecylsulfate polyacrylamide
gel electrophoresis (SDS PAGE)
and fluorography

Introduction

Sodium dodecylsulfate polyacrylamide gel electrophoresis is a very powerful technique used to analyze a complicated mixture of polypeptides. SDS PAGE permits the determination of the molecular weights of these polypeptides by comparing them to protein standards run on the same gel. For an excellent discussion of electrophoretic theory and the application of SDS PAGE, see your Hoefer Catalog, pages 128-134.

Our present task is to determine the molecular weights of the proteins translated from the Codium fragile mRNA in the wheat germ lysate translation system. These proteins were not generated in sufficient quantities to visualize in a gel by conventional staining methods, so we must rely on the ^{35}S -methionine incorporated into these polypeptides to aid in their characterization. As the ^{35}S decays, it will expose a piece of x-ray film placed against the dried gel. The beta particles, however, do not possess sufficient energy to expose the film directly. To solve this problem, the gels are impregnated with a fluor (hence fluorography) that shifts the particle wavelength to a wavelength of light that x-ray film is sensitive to. Several products are commercially available for this purpose, such as Amplify (Amersham) and En- $^3\text{Hance}$ (DuPont); homemade fluors like DMSO/PPO are common. Unfortunately, all these methods are fairly noxious and not much fun. We will use sodium salicylate, which is relatively safe and rapid and produces good results.

Several things require your attention. First of all, unpolymerized acrylamide is a neurotoxin and must be handled with care. Wear gloves and use a dust mask while weighing acrylamide out. Acrylamide is absorbed through bare skin, so avoid accidental contact and spillage. Secondly, remember that the samples used are radioactive. Handle these with care. The unincorporated ^{35}S -methionine present in the samples will contaminate the electrophoresis running buffers. Dispose of these cautiously. Finally, and most importantly, ensure that your gels are absolutely, completely and thoroughly dry before applying any film, or unpleasant things will happen.

Materials

Vertical slab gel electrophoresis rig and accessories

Shaker table

Pans to hold gels

Power supply

Filter paper

Gel dryer

8" x 10" cardboard

XAR-X-Ray film, Cassette, Intensifying Screen

D-11 developer, stop, fixer

-70°C freezer

Whatman 3 MM filter paper

Solutions

PAGE Solutions:

See Hoefer catalog pages 131-133 for the required solutions. Initially, these will be provided.

Sample buffer

6.25 mM Tris, pH 6.8	0.38 g
10% glycerol	5.00 g
5% 2-mercaptoethanol	2.50 ml
2.3% SDS	1.15 g
0.1% Bromophenol blue	50 mg
H ₂ O	to 50 ml

Adjust pH to 6.8 with HCl prior to adding the mercaptoethanol. Store at 4°C.

Gel fixative

8.5% acetic acid	42.5 ml glacial acetic acid
25% ethanol	132 ml 95% ethanol
H ₂ O	to 500 ml

Fluorography solution

1.0 M Na salicylate	80.05 g
H ₂ O	to 500 ml

Developer & Fixer

Prepare following package instructions.

Procedure

Gels may be poured ahead of time and stored for several days at 4°C

1 mm spacers

*Also run 100,000 cpm
+ background translations!*

<u>Marker</u>	<u>Mol. weight</u>
lysozyme	14,300
carbonic anhydrase	30,000
ovalbumin	46,000
bovine serum albumin	69,000
phosphorylase b	92,500
myosin	200,000

Allow 3 to 5 hours for gels to run.

30 mAmps per gel; 10°C

The tank buffer may be radioactive.
Dispose of correctly.

Your friendly neighborhood T.A. will demonstrate.

1. Set up the gel apparatus and pour a 10% acrylamide, 0.1% SDS gel. Use 1.0 mm thick spacers. Follow Pharmacia's instructions for setting up the equipment, and Hoefer's instructions for pouring the gels.

2. Remove 100,000 TCA precipitable cpm's from the Codium and background translations. Add 20-25 μ l of sample buffer, but do not exceed 35 μ l total volume. Use Eppendorf tubes.

3. Remove 1.0 μ l of ^{14}C -methylated protein molecular weight markers from a stock aliquot (stored at -20°C). Add 20-25 μ l of sample buffer.

4. Place the samples and markers into a boiling water bath and heat for 2 minutes.

5. Remove all samples and allow to cool.

6. Load gel using a 50 μ l Hamilton-type syringe. Fill the unused wells with 30 μ l of sample buffer.

7. Make final assembly of gel apparatus and run at about 12 milliamps per gel in a constant current mode.

8. At completion of electrophoresis, disassemble the gel apparatus. Carefully separate the glass sandwich.

9. Using a ruler with a metal edge, trim the gel to size by cutting down directly through the gel. Discard the excess portions. Cut off the lower left hand corner of the gel for orientation.

These gels are very fragile and tear easily. Acrylamide gels do not make good jigsaw puzzles.

It may be easier to slide the filter paper under the gel while it is still in the Na salicylate solution.

It is imperative that the gel be absolutely dry, otherwise it will stick to the film and ruin all your work. Dry the gel overnight if necessary.

at least 2 hours
polyethylene sheet
wrap
gel
filter paper
filter paper
steel

If there is no red light available, this must be performed in total darkness.

Make sure that both the trimmed corners (gel and film) are on the same side.

Wrong

10. Very carefully remove the gel from the glass plate and place it into a dish of gel fixative. Shake gently using the shaker table at RT for 30 minutes.

11. Wash gel in distilled water: 3 changes in 15 minutes. Continue to agitate gel.

12. Cover the gel with 1.0 M Na salicylate and agitate for 30 minutes.

13. Remove the gel and place it on a piece of Whatman 3 mm filter paper cut somewhat larger than the gel. Be careful. Avoid trapping bubbles between the gel and the paper.

14. Carefully Place the gel and filter paper on the dryer and cover it with Saran Wrap. Dry the gel following the instrument's instructions.

15. Remove the gel from the dryer and tape it down securely to an 8 x 10 piece of cardboard.

16. In the darkroom, and under a red safety light, remove a piece of film from the box. Cut off the lower left hand corner of the film.

17. Place the gel inside the cardboard cassette. Lay the film over the gel, then place the intensifying screen - taped side down - over the film.

18. Close up the cassette and wrap the package inside a sheet of aluminum foil. Clamp everything in place with 2 bulldog clamps.

Try not to let the film/gel package warm significantly.

Return photographic solutions to their stock bottles.

Allow the gel to warm to room temperature before applying new film.

19. Place the package in the -70°C freezer for 16-24 hours.

20. Set up the D-11 developer, stop bath (1.0% acetic acid), and fixative in the darkroom.

21. Under the red light, or total darkness, quickly remove the film from the cold package.

22. Develop the film:

- a) D-11 for 3 minutes.
- b) Stop both for 30 seconds.
- c) Fixer for 3 minutes.
Agitate every 30 seconds.
- d) Rinse in H₂O.

23. Examine your fluorograph. Fresh film may be re-applied to the gel if a longer or shorter exposure is required.

24. Using the molecular weight markers and the instructions in the Hoefer catalog, you may calculate the molecular weights of the Codium proteins. The presence of high molecular weight proteins (above 75,000) indicates that the purified mRNA is reasonably intact and that its preparation was a good one.

References

Hoefler Scientific Instruments 1988-1989 Catalog, pp 128-134.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. Nature 227:680-685.

Hoefler Scientific Instruments

1988-1989

1988-1989

1988-1989

1988-1989

1988-1989

1988-1989

1988-1989

Gels

1.0 mm thick spacers

	10% Separating	5% Stacking	4% Stacking
H ₂ O	16.07	11.37	12.20
Buffer	10.00	5.00	5.00
acrylamide	13.33	3.33	2.66
10% SDS	0.40	0.20	0.20
10% APS	200ul	100ul	100ul
TEMED	<u>133ul</u>	<u>10ul</u>	<u>10ul</u>
	40ml	20ml	20ml

1mm thick Hoefer gels

	10%	Separating	5%	Stacking
Buffer	4.4	8.8	2.5 ml	5.0
H ₂ O	7.5	15.0	5.92	11.84
36% Acryl.	5.85	11.7	1.58	3.16
10% APS	30 μ l	60 μ l	30 μ l	60 μ l
TEMED	15 μ l	30 μ l	15 μ l	30 μ l

Stuff in lab

Glass plates
Spacers and combs (~~Scientific Products no. 0144444444~~)
Silastic tubing
Clamps
Gel rigs (shop made or Hoeffer)
Power packs

SOLUTIONS

30%(w/w) Acrylamide

		Final Conc.
Acrylamide	58.4 g.	29.2%
Bis-acrylamide	1.6 g.	0.8%
H ₂ O	to 200 g.	

Note! This solution is weight to weight. Filter through Whatman No. 1 and store in an amber bottle at 4 C.

Separating Gel Buffer, pH 8.8

		Final Conc.
Tris	36.33 g.	1.5M
SDS	0.80 g.	0.4%
H ₂ O	to 200 ml.	

Adjust pH to 8.8 with HCl. Store at 4 C.

Stacking Gel Buffer, pH 6.8

		Final Conc.
Tris	6.06 g.	0.5M
SDS	0.40 g.	0.4%
H ₂ O	to 100 ml.	

Running Buffer, 10X Stock (Large Gel Apparatus)

		Final Conc.
Tris	30.30 g.	0.025M
Glycine	144.00 g.	0.192M
SDS	10.00 g.	0.1%
H ₂ O	to 1000 ml.	

Dilute 100 ml. to 1.0 liter with distilled H₂O prior to use. Concentrations are for final solution.

Running Buffer for the Hoeffer Rig

			Final Conc.
Tris	3.0 g/liter	54.0 g.	0.025M
Glycine	14.4 g/liter	259.2 g.	0.192M
SDS	1.0 g/liter	18.0 g.	0.1%
H ₂ O		to 18.0 liters	

Due to the large volume of this solution, prepare it with tap distilled water. Our still couldn't keep up

with this kind of demand. Store the solution in the large plastic carboy used for this purpose. Keep the solution at 4 C. Replace after 12 gels.

Sample Buffer, pH 6.8

		Final Conc.
Tris	0.38 g.	0.0625M
Glycerol	5.00 g.	10%
2-ME	2.50 ml.	5%
SDS	1.15 g.	2.3%
Bromphenol Blue	50 mg.	0.1%
H ₂ O	to 50 ml.	

Adjust pH to 6.8 with HCl prior to adding 2-mercaptoethanol. Store at 4C. Bromphenol blue may be added now, or a 0.1% stock solution can be prepared. A small amount (2-4 ul) can be added to the samples for visualization before loading the gel.

Stain 1

Brilliant
0.2% Coomassie Blue R-250
25% Methanol
10% Acetic Acid
H₂O

1.0 g.
225 ml. 125 ml
50 ml.
to 500 ml. →

Filter through Whatman No. 1. Stain gel for one hour.

Stain 2

Brilliant
0.05% Coomassie Blue R-250
25% Isopropanol
10% Acetic Acid
H₂O

250 mg.
125 ml.
50 ml.
to 500 ml.

Stain gels overnight.

De-stain 1

30% Methanol
10% Acetic Acid

Use this solution to de-stain gels stained with stain solution 1. De-stain for 2-3 hours. Replace stain with 10% acetic acid to maintain color of bands.

De-stain 2

25% Isopropanol
10% Acetic Acid

Use this de-staining solution on gels stained with stain 2. Watch the de-staining process carefully since it is very easy to destain the bands as well. As above, replace

SDS GELS

Electrophoretic transfers are much faster than Southern blots, taking from one-half to two hours to perform. The gel, containing protein or nucleic acids, is placed next to a membrane in a gel-holding cassette and then placed in a tank filled with buffer. An electric field is applied perpendicular to the cassette and the sample moves out of the gel and onto the membrane. The result is an exact copy of the original gel.

By either method, the sample moves from the gel matrix to the

surface of the membrane. Once the sample is bound to the membrane, it is detected in one of several methods. If the sample itself is radioactive, the membrane can be subjected directly to autoradiography. If it is not radioactive, radioactive probes such as complementary DNA or specific antibodies can be bound either to specific nucleic acids sequences or to specific protein antigens. The membrane is then subjected to autoradiography to detect the positions of the probes.

Systems and Exercises

The exercises which follow are examples of the most commonly used electrophoretic systems and techniques. They are presented step by step so that a newcomer to electrophoresis can easily perform a separation or analysis with no additional reading or instruction.

The samples used in these exercises are merely examples of what can be separated in each system. Other sample mixtures work equally well.

Separation of Proteins on the Basis of Molecular Weight: SDS Gel Electrophoresis

In SDS separations, migration is determined not by intrinsic electrical charge of polypeptides but by molecular weight (Shapiro, 1967). Sodium dodecylsulfate (SDS) is an anionic detergent which denatures proteins by wrapping around the polypeptide backbone. In so doing, SDS confers a net negative charge to the polypeptide in proportion to its length. When treated with SDS and a reducing agent, the polypeptides become rods of negative charges with equal "charge densities" or charge per unit length.

The major usefulness of this system is to determine the molecular

weights of polypeptides. This is done by running a gel with standard proteins of known molecular weights along with the polypeptide to be characterized. A linear relationship exists between the \log_{10} of the molecular weight of a polypeptide and its R_f . By measuring R_f , that is, the distance from the top of the gel to the polypeptide divided by the distance from the top of the gel to the dye front, a standard curve can be generated. The curve will show the R_f of the standard polypeptides and the \log_{10} of their molecular weights. The R_f of the polypeptide to be characterized is determined in the same way, and the \log_{10} of its molecular weight read directly from the standard curve; the antilog is the molecular weight.

There are two SDS systems commonly used today. The Weber and Osborn (1969) system is a continuous system and is relatively easy to set up. The Laemmli system (1970), a modification of Ornstein (1964) and Davis (1964), is a discontinuous SDS system (see page 131) and is probably the most widely used electrophoretic system today. The treated peptides are stacked in a stacking gel before entering the separating gel, and hence, the resolution in a Laemmli gel is excellent.

EXERCISE

Determination of the Molecular Weights of E. coli Proteins By SDS Gel Electrophoresis

Equipment

SE 600 Vertical Slab Unit (page 11)
PS 500XT Power Supply (page 60)
SE 530 Slab Gel Diffusion Destainer (page 19)
PR 70 Red Rotor (page 105)
SY 550 50 μ l Syringe (page 18)
MS 66 Spin Stir Magnetic Stirrer (page 106)

Stock Solutions

(All solutions should be filtered.)

Use the free base forms of tris and glycine when making these solutions. The term "tris-HCl" used in these formulations, is an indication that the pH of the solution is adjusted with HCl.

1. Monomer Solution

(30%T 2.7%C_W)

Acrylamide 58.4 g

ACRYLAMIDE IS NEUROTOXIC AND SHOULD BE HANDLED WITH CARE.

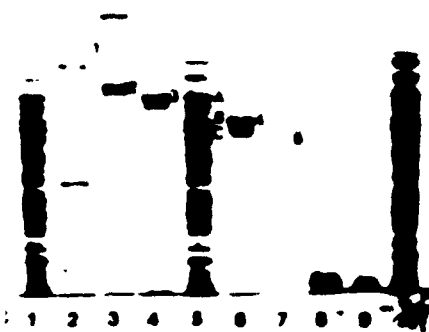
Bis 1.6 g

H₂O to 200 ml

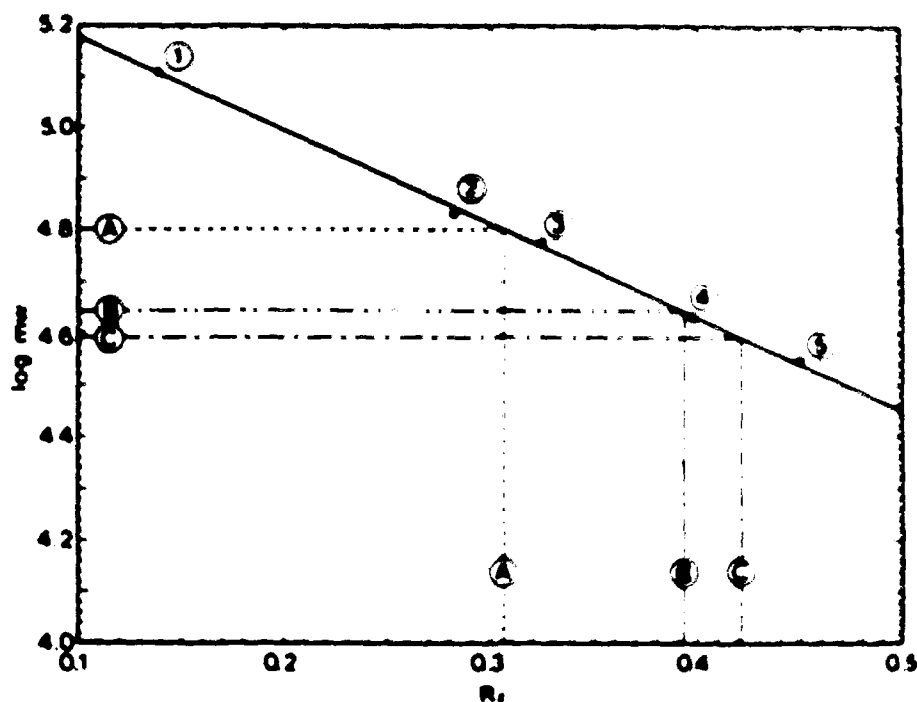
Store at 4°C in the dark.

SDS GELS

Protein	Molecular Weight	Log ₁₀ MW	Distance to Band	Unknown Proteins			
				R _f (Dist 33.8)	R _f	Log ₁₀ MW	Molecular Weight
1. β -Galactosidase	130,000	5.114	4.7	0.29	0.29	5.114	130,000
2. Bovine Serum Albumin	68,000	4.833	9.6	0.34	0.34	4.833	68,000
3. Catalase	60,000	4.778	11.2	0.37	0.37	4.778	60,000
4. Ovalbumin	44,000	4.643	13.2	0.39	0.39	4.643	44,000
5. Pepsin	35,000	4.544	15.2	0.41	0.41	4.544	35,000



12%T 2.7%C SDS gel for the determination of the molecular weights of polypeptides
 Lanes 1 & 10: 40 μ g E. coli proteins. 2: 15 μ g β -galactosidase (130,000 daltons). 3: 15 μ g bovine serum albumin (68,000 daltons). 4: 15 μ g catalase (60,000 daltons). 5: 15 μ g ovalbumin (44,000 daltons). 6: 15 μ g pepsin (35,000 daltons). 7: 15 μ g myoglobin (17,000 daltons). 8: 15 μ g lysosyme (14,400 daltons).



Standard curve for the determination of the molecular weights of polypeptides The standard curve was generated by plotting the R_f of each standard protein against the log₁₀ of its molecular weight. The molecular weight of an unknown polypeptide can then be determined by finding its R_f on the standard curve and reading the log₁₀ MW from the ordinate. The antilog of this number is the molecular weight of the polypeptide.

2. 4X Running Gel Buffer

(0.5 M Tris-Cl pH 8.8)

Tris 36.3 g
 Adjust to pH 8.8 with HCl
 H₂O to 200 ml

3. 4X Stacking Gel Buffer

(0.5 M Tris-Cl pH 6.8)

Tris 4.0 g
 Adjust to pH 6.8 with HCl
 H₂O to 50 ml

4. 10% SDS

SDS 50 g
 H₂O to 500 ml

5. Initiator

(10% ammonium persulfate)

Ammonium persulfate 0.5 g
 H₂O to 5.0 ml

6. Running Gel Overlay

(0.375 M Tris-Cl pH 8.8, 0.1% SDS)

Tris 25 ml Solution (2%)
 SDS 1.0 ml Solution (4%)
 H₂O to 100 ml

7. 2X Treatment Buffer

(0.125 M Tris-Cl pH 6.8, 4% SDS)

20% glycerol 10% 2-mercaptoethanol
 Tris 2.5 ml Solution (3%)
 SDS 4.0 ml Solution (4%)

Glycerol 2.5 ml
 2-mercaptoethanol 1.0 ml
 H₂O to 100 ml
 Include in aliquots and freeze.

8. Tank Buffer

(0.025 M Tris pH 8.8, 0.012 M glycine, 0.1% SDS)

Tris 12.5 g
 Glycine 57.5 g
 SDS 4.0 ml Solution (4%)
 H₂O to 400 ml

Because the pH of this solution need not be checked, it can be

SDS GELS

made up directly in large reagent bottles marked in 10 liters. 12.5 liters can be made up at a time. Reuse the buffer in the lower buffer chamber 4 or 5 times, but discard the upper buffer after each run.

9. Stain Stock

(1% Coomassie Blue R 250)
Coomassie Blue R 250 2.5 g
H₂O 200 ml
Stir and filter.

10. Stain

(0.125% Coomassie Blue R 250)
50% methanol, 10% acetic acid
Coomassie Blue R 250 2.5 ml
Stain Stock (9)
Methanol 250 ml
Acetic acid 50 ml
H₂O to 500 ml

11. Destaining Solution I

(50% methanol, 10% acetic acid)
Methanol 500 ml
Acetic acid 100 ml
H₂O to 1.0 liter

12. Destaining Solution II

(70% acetic acid, 5% methanol)
Acetic acid 700 ml
Methanol 50 ml
H₂O to 1.0 liters

13. Water-Saturated n-Butanol

n-butanol 50 ml
H₂O 5 ml

Combine in a bottle and shake. Use the top phase to overlay your gels.

Procedure

Preparation of the Separating Gel

1. Assemble the SE 600 Vertical Slab Gel Unit in the casting mode. Use 1.5 mm spacers.
2. In a 125 ml side arm vacuum flask, mix 60 ml of separating gel solution according to Table I. Leave out the ammonium persulfate and the TEMED. Add a small magnetic stir bar.
3. Stopper the flask and apply a vacuum for several minutes while stirring on a magnetic stirrer.

4. Add the TEMED and ammonium persulfate and gently swirl the flask to mix. Be careful not to generate bubbles.

5. Pipet the solution into the sandwich to a level about 4.0 cm from the top.

6. Overlay the slabs. Use a lightly greased 1 ml glass syringe fitted with a 2 inch 22 gauge needle. Position the needle, bevel up, at about a 45° angle so that the point is at the top of acrylamide and next to a spacer. Gently apply about 0.3 ml of water or water saturated n-butanol. Repeat on the other side of the slab next to the other spacer. The water will layer evenly across the entire surface after a minute or two. Overlay the second slab in the same manner. A very sharp liquid gel interface will be visible when the gel is polymerized.

7. Tilt the casting stand to pour off the overlay.

8. Rinse the surfaces of the gels once with distilled water.

9. Add about 1.0 ml of Running Gel Overlay Solution (6).

10. Allow the gels to sit for several hours.

Preparation of the Stacking Gel

11. Pour the liquid from the surface of the gels.

12. In a 50 ml side arm vacuum flask, mix 20 ml of stacking gel solution according to Table I. Leave out the ammonium persulfate and the TEMED. Add a magnetic stir bar.

13. Deaerate the solution as before.

14. Add the ammonium persulfate and TEMED. Gently swirl the flask to mix.

15. Add 1-2 ml of stacking gel solution to each sandwich to rinse the surface of the gels. Rock the casting stand and pour off the liquid.

16. Fill each sandwich with stacking gel solution.

TABLE I

	Separating Gel	Stacking Gel
	10%T 2.7%C	4%T 2.7%C
30%T 2.7%C	20 ml	2.66 ml
Buffer (2)	15 ml	—
Buffer (3)	—	5.0 ml
10% SDS (4)	0.6 ml	0.2 ml
H ₂ O	24.4 ml	12.2 ml
Ammonium persulfate (5)	300 µl	100 µl
TEMED	20 µl	10 µl



SDS gel run with the lower buffer chamber filled high enough just to make contact with the bottom of the slab. Note the curved (smiling) appearance of the gel due to heating in the center of the gel. Compare this gel with the gel in photo 1 where the lower buffer chamber was filled to cover the slabs completely.

17. Insert a comb into each sandwich. Take care not to trap any bubbles below the teeth of the combs. Oxygen will inhibit polymerization and will cause a local distortion in the gel surface at the bottom of the wells.
18. Allow the gel to sit for at least a half hour.
19. Combine equal parts of protein sample and 2X Treatment Buffer (7) in a test tube.
20. Put the tube in a boiling water bath for 90 seconds.
21. Remove the sample and put it on ice until ready to use. This treated sample can be put in the freezer for future runs.

SDS GELS

Loading and Running the Gels

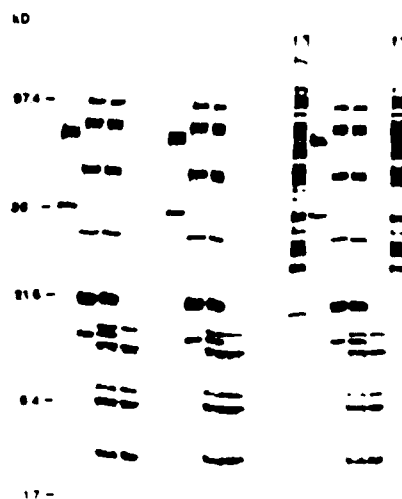
22. Slowly remove the combs from the gels. Be careful to pull the comb straight up to avoid disturbing the well dividers.
 23. Rinse each well with distilled water.
 24. Invert the casting stand to drain the wells.
 25. Right the casting stand.
 26. Fill each well with tank buffer.
 27. Using a 50 μ l syringe, underlay the sample in each well.
 28. Put the upper buffer chamber in place. Remove lower cams and cam the sandwiches to the bottom of the upper buffer chamber. Put the upper buffer chamber in place on the heat exchanger in the lower buffer chamber.
 29. Fill the lower buffer chamber with Tank Buffer (8) until the sandwiches are immersed in buffer. If bubbles get trapped under the ends of the sandwiches, coax them away with a pipet.
 30. Add a spinbar to the lower buffer chamber and place the chamber on a magnetic stirrer. When the lower buffer is circulated, the temperature of the buffer remains uniform. This is important, because uneven heating distorts the banding pattern of the gel.
 31. Put a drop of 0.1% phenol red in the upper buffer chamber. This is the tracking dye. Alternatively, add the dye directly to the sample after it has been heat treated.
 32. Fill the upper buffer chamber with Tank Buffer (8). Take care not to pour buffer into the sample wells because it will wash the sample out.
 33. Put the lid on the unit and connect it to the PS 500XT Power Supply. The cathode should be connected to the upper buffer chamber.
 34. Set the power supply to constant current.
 35. Turn the power supply on and adjust the current to 30 ma/1.5 mm thick gel. If you have two 1.5 mm gels you should adjust the power supply to 60 ma; if you have one 1.5 mm gel and one 0.75 mm gel, the setting should be 45 ma. The voltage should start at about 70-80 V, but will increase during the run. Keep a record of the voltage and current readings so that future runs can be compared. In this way, you will easily be able to detect current leaks or incorrectly made buffers.
 36. When the gel reaches the bottom, about five hours later, turn the power supply off and disconnect the power cables. If you want to run the gel for a longer period, say for ten hours, cut the current in half to 15 ma/1.5 mm gel; for 15 hours, cut the current to 10 ma/1.5 mm gel.
- ### Staining and Destaining the Gels
37. Disassemble the sandwiches and put the gels into Stain (10).
 38. Gently shake the gels for 4-8 hours on the PR 70 Red Rotor.
 39. Remove the gels and put them in Destaining Solution I (11). Shake for one hour.
 40. Transfer the gels to the SE 530 Destainer filled with Destaining Solution II (12).

EXERCISE

The Electrophoretic Separation of Low Molecular Weight Polypeptides in Polyacrylamide Gels

Contributed by Gary Guller and Dr. James Graham, Department of Physiology, University of Wisconsin, Madison, Wisconsin

The most commonly used protein electrophoresis system in use today is that described by Laemmli (1970). Laemmli gels, by including sodium dodecyl sulfate (SDS) in the gel formulation (separating gel, 2% cross linking, pH 8.8) and by treating the proteins with SDS and reducing agent, separate polypeptides on the basis of molecular weight alone. The effects of intrinsic charge and native conformation become negligible



Low molecular weight polypeptide SDS separation.

The gel shown is a 4-20% gradient gel. The lanes contain: Lane 1: Molecular weight markers (10, 97.4, 66, 44, 33, 22 kDa). Lane 2: BSA (66 kDa). Lane 3: Pepsin (36 kDa). Lane 4: Trypsin (24 kDa). Lane 5: Chymotrypsin (24 kDa). Lane 6: Elastase (21 kDa). Lane 7: Cathepsin B (23 kDa). Lane 8: Cathepsin D (28 kDa). Lane 9: Cathepsin E (23 kDa). Lane 10: Cathepsin G (24 kDa). Lane 11: Cathepsin H (23 kDa). Lane 12: Cathepsin L (23 kDa). Lane 13: Cathepsin S (23 kDa). Lane 14: Cathepsin V (23 kDa). Lane 15: Cathepsin W (23 kDa). Lane 16: Cathepsin X (23 kDa). Lane 17: Cathepsin Y (23 kDa). Lane 18: Cathepsin Z (23 kDa). Lane 19: Cathepsin A (23 kDa). Lane 20: Cathepsin B (23 kDa). Lane 21: Cathepsin C (23 kDa). Lane 22: Cathepsin D (23 kDa). Lane 23: Cathepsin E (23 kDa). Lane 24: Cathepsin F (23 kDa). Lane 25: Cathepsin G (23 kDa). Lane 26: Cathepsin H (23 kDa). Lane 27: Cathepsin I (23 kDa). Lane 28: Cathepsin J (23 kDa). Lane 29: Cathepsin K (23 kDa). Lane 30: Cathepsin L (23 kDa). Lane 31: Cathepsin M (23 kDa). Lane 32: Cathepsin N (23 kDa). Lane 33: Cathepsin O (23 kDa). Lane 34: Cathepsin P (23 kDa). Lane 35: Cathepsin Q (23 kDa). Lane 36: Cathepsin R (23 kDa). Lane 37: Cathepsin S (23 kDa). Lane 38: Cathepsin T (23 kDa). Lane 39: Cathepsin U (23 kDa). Lane 40: Cathepsin V (23 kDa). Lane 41: Cathepsin W (23 kDa). Lane 42: Cathepsin X (23 kDa). Lane 43: Cathepsin Y (23 kDa). Lane 44: Cathepsin Z (23 kDa). Lane 45: Cathepsin A (23 kDa). Lane 46: Cathepsin B (23 kDa). Lane 47: Cathepsin C (23 kDa). Lane 48: Cathepsin D (23 kDa). Lane 49: Cathepsin E (23 kDa). Lane 50: Cathepsin F (23 kDa). Lane 51: Cathepsin G (23 kDa). Lane 52: Cathepsin H (23 kDa). Lane 53: Cathepsin I (23 kDa). Lane 54: Cathepsin J (23 kDa). Lane 55: Cathepsin K (23 kDa). Lane 56: Cathepsin L (23 kDa). Lane 57: Cathepsin M (23 kDa). Lane 58: Cathepsin N (23 kDa). Lane 59: Cathepsin O (23 kDa). Lane 60: Cathepsin P (23 kDa). Lane 61: Cathepsin Q (23 kDa). Lane 62: Cathepsin R (23 kDa). Lane 63: Cathepsin S (23 kDa). Lane 64: Cathepsin T (23 kDa). Lane 65: Cathepsin U (23 kDa). Lane 66: Cathepsin V (23 kDa). Lane 67: Cathepsin W (23 kDa). Lane 68: Cathepsin X (23 kDa). Lane 69: Cathepsin Y (23 kDa). Lane 70: Cathepsin Z (23 kDa). Lane 71: Cathepsin A (23 kDa). Lane 72: Cathepsin B (23 kDa). Lane 73: Cathepsin C (23 kDa). Lane 74: Cathepsin D (23 kDa). Lane 75: Cathepsin E (23 kDa). Lane 76: Cathepsin F (23 kDa). Lane 77: Cathepsin G (23 kDa). Lane 78: Cathepsin H (23 kDa). Lane 79: Cathepsin I (23 kDa). Lane 80: Cathepsin J (23 kDa). Lane 81: Cathepsin K (23 kDa). Lane 82: Cathepsin L (23 kDa). Lane 83: Cathepsin M (23 kDa). Lane 84: Cathepsin N (23 kDa). Lane 85: Cathepsin O (23 kDa). Lane 86: Cathepsin P (23 kDa). Lane 87: Cathepsin Q (23 kDa). Lane 88: Cathepsin R (23 kDa). Lane 89: Cathepsin S (23 kDa). Lane 90: Cathepsin T (23 kDa). Lane 91: Cathepsin U (23 kDa). Lane 92: Cathepsin V (23 kDa). Lane 93: Cathepsin W (23 kDa). Lane 94: Cathepsin X (23 kDa). Lane 95: Cathepsin Y (23 kDa). Lane 96: Cathepsin Z (23 kDa). Lane 97: Cathepsin A (23 kDa). Lane 98: Cathepsin B (23 kDa). Lane 99: Cathepsin C (23 kDa). Lane 100: Cathepsin D (23 kDa). Lane 101: Cathepsin E (23 kDa). Lane 102: Cathepsin F (23 kDa). Lane 103: Cathepsin G (23 kDa). Lane 104: Cathepsin H (23 kDa). Lane 105: Cathepsin I (23 kDa). Lane 106: Cathepsin J (23 kDa). Lane 107: Cathepsin K (23 kDa). Lane 108: Cathepsin L (23 kDa). Lane 109: Cathepsin M (23 kDa). Lane 110: Cathepsin N (23 kDa). Lane 111: Cathepsin O (23 kDa). Lane 112: Cathepsin P (23 kDa). Lane 113: Cathepsin Q (23 kDa). Lane 114: Cathepsin R (23 kDa). Lane 115: Cathepsin S (23 kDa). Lane 116: Cathepsin T (23 kDa). Lane 117: Cathepsin U (23 kDa). Lane 118: Cathepsin V (23 kDa). Lane 119: Cathepsin W (23 kDa). Lane 120: Cathepsin X (23 kDa). Lane 121: Cathepsin Y (23 kDa). Lane 122: Cathepsin Z (23 kDa). Lane 123: Cathepsin A (23 kDa). Lane 124: Cathepsin B (23 kDa). Lane 125: Cathepsin C (23 kDa). Lane 126: Cathepsin D (23 kDa). Lane 127: Cathepsin E (23 kDa). Lane 128: Cathepsin F (23 kDa). Lane 129: Cathepsin G (23 kDa). Lane 130: Cathepsin H (23 kDa). Lane 131: Cathepsin I (23 kDa). Lane 132: Cathepsin J (23 kDa). Lane 133: Cathepsin K (23 kDa). Lane 134: Cathepsin L (23 kDa). Lane 135: Cathepsin M (23 kDa). Lane 136: Cathepsin N (23 kDa). Lane 137: Cathepsin O (23 kDa). Lane 138: Cathepsin P (23 kDa). Lane 139: Cathepsin Q (23 kDa). Lane 140: Cathepsin R (23 kDa). Lane 141: Cathepsin S (23 kDa). Lane 142: Cathepsin T (23 kDa). Lane 143: Cathepsin U (23 kDa). Lane 144: Cathepsin V (23 kDa). Lane 145: Cathepsin W (23 kDa). Lane 146: Cathepsin X (23 kDa). Lane 147: Cathepsin Y (23 kDa). Lane 148: Cathepsin Z (23 kDa). Lane 149: Cathepsin A (23 kDa). Lane 150: Cathepsin B (23 kDa). Lane 151: Cathepsin C (23 kDa). Lane 152: Cathepsin D (23 kDa). Lane 153: Cathepsin E (23 kDa). Lane 154: Cathepsin F (23 kDa). Lane 155: Cathepsin G (23 kDa). Lane 156: Cathepsin H (23 kDa). Lane 157: Cathepsin I (23 kDa). Lane 158: Cathepsin J (23 kDa). Lane 159: Cathepsin K (23 kDa). Lane 160: Cathepsin L (23 kDa). Lane 161: Cathepsin M (23 kDa). Lane 162: Cathepsin N (23 kDa). Lane 163: Cathepsin O (23 kDa). Lane 164: Cathepsin P (23 kDa). Lane 165: Cathepsin Q (23 kDa). Lane 166: Cathepsin R (23 kDa). Lane 167: Cathepsin S (23 kDa). Lane 168: Cathepsin T (23 kDa). Lane 169: Cathepsin U (23 kDa). Lane 170: Cathepsin V (23 kDa). Lane 171: Cathepsin W (23 kDa). Lane 172: Cathepsin X (23 kDa). Lane 173: Cathepsin Y (23 kDa). Lane 174: Cathepsin Z (23 kDa). Lane 175: Cathepsin A (23 kDa). Lane 176: Cathepsin B (23 kDa). Lane 177: Cathepsin C (23 kDa). Lane 178: Cathepsin D (23 kDa). Lane 179: Cathepsin E (23 kDa). Lane 180: Cathepsin F (23 kDa). Lane 181: Cathepsin G (23 kDa). Lane 182: Cathepsin H (23 kDa). Lane 183: Cathepsin I (23 kDa). Lane 184: Cathepsin J (23 kDa). Lane 185: Cathepsin K (23 kDa). Lane 186: Cathepsin L (23 kDa). Lane 187: Cathepsin M (23 kDa). Lane 188: Cathepsin N (23 kDa). Lane 189: Cathepsin O (23 kDa). Lane 190: Cathepsin P (23 kDa). Lane 191: Cathepsin Q (23 kDa). Lane 192: Cathepsin R (23 kDa). Lane 193: Cathepsin S (23 kDa). Lane 194: Cathepsin T (23 kDa). Lane 195: Cathepsin U (23 kDa). Lane 196: Cathepsin V (23 kDa). Lane 197: Cathepsin W (23 kDa). Lane 198: Cathepsin X (23 kDa). Lane 199: Cathepsin Y (23 kDa). Lane 200: Cathepsin Z (23 kDa).

Unit 8.

Preparation of a crude protein
extract from Codium fragile

Introduction

Algal tissues present several problems in extracting proteins suitable for analysis by SDS PAGE. Polyphenols and polysaccharides present in algal tissues create several difficulties that must be overcome during an extraction procedure. Polyphenols polymerize or oxidize into other compounds that inhibit enzymatic activities and perhaps alter other characteristics of the proteins as well. The major source of problems, however, stems from the polysaccharides. Acidic polysaccharides, released upon homogenization of the algal tissue, alter the pH of the extraction environment and cause the denaturation of enzymes and the elimination of their activities. Alginates, polysaccharide components of algal cell walls and intercellular spaces, create a viscous solution when released, complicating the extraction procedure. Finally, the polysaccharides in general purify along with the proteins and will dramatically distort banding patterns on SDS gels, making the gels largely useless. Simply put, polysaccharides gum up the works.

The primary weapon available to combat these difficulties is the extraction buffer. Its composition is critical to a successful extraction. First of all, the addition of a detergent, namely Tween 80, greatly improves the protein yield by disrupting membranes and improving protein solubility. MOPS, a Goode biological buffer, has the buffering capacity to overcome the pH changes caused by the acidic polysaccharides. The reducing agents dithiothreitol (DTT) and sodium ascorbate prevent the oxidation of the polyphenols. Additionally, the polyphenols bind to polyvinyl polypyrrolidone. This insoluble compound is easily removed from the extraction buffer by filtration and centrifugation, bringing the polyphenols along with it.

Alginates will precipitate when complexed with calcium ions added as CaCl_2 and then chelated with EDTA. Taken all together, the extraction buffer is a mixture of buffers, reducing agents, salts and detergent. Each component works in concert with the others to attack an onslaught of potential disasters.

Polysaccharides will continue to provide problems, and consequently require additional tactics. After a centrifugation spin to pellet those compounds that have precipitated in the extraction buffer, the proteins and some polysaccharides are removed from the extraction buffer itself by ammonium sulfate precipitation. Ion exchange chromatography on DEAE cellulose further fractionates the proteins and remaining polysaccharides. Both bind to DEAE cellulose; by adjusting the ionic strength of the column buffers, the proteins will elute, leaving the polysaccharides behind.

The proteins finally obtained, when carefully concentrated, will run on an SDS polyacrylamide gel and show nice banding patterns when stained with Coomassie Blue.

The procedure we will use is not difficult, however, it is still exploratory. A considerable amount of work still needs to be done to determine the ideal conditions for protein extraction from algae. This is particularly important because any differences found in enzyme levels or isozyme patterns are likely to be artifactual.

If we are successful with the protein extracts, our next goal is to run gels and then electrophoretically transfer the proteins (Western blotting) to nitrocellulose membranes. We will probe the nitrocellulose blots with our anti-Codium fragile lectin antibody, and hunt down glycoproteins with the lectin Concanavalin A.

Materials

Fresh Codium fragile

Liquid nitrogen

mortar and pestle

beakers - 100 ml

stirbars and stirring plates

Miracloth

DEAE cellulose and columns

Related column supplies

Fraction collector and tubes

spectrophotometer

Quartz cuvettes

Acetone

Cold room

Clamps

Miracloth

Funnels

30 ml Corex tubes

50 ml polypropylene tubes

Vertical slab gel equipment

Solutions and Reagents

1) Polyvinylpyrrolidone

(Prepared according to Loomis, 1974)

- A. Pour 50 g of PVPP into 250 ml of 10% HCl.
- B. Boil for 10 min.
- C. Transfer to a Buchner funnel and wash with distilled water. Use about 1 liter.
- D. Return the PVPP to a beaker and resuspend into a slurry in water.
- E. Add 1.0 M KOH (carefully!) to bring the pH to about 7, as measured by the pH meter.
- F. Return to the Buchner funnel and continue washing with water.
- G. Remove the excess water and store the washed PVPP wet at 4°C.

2) Extraction buffer

50 mM MOPS, pH 7.4	2.10 g	2.10 g
2 mM EDTA	148.88 mg	26.0 mg
50 mM Na Ascorbate	1.98 g	2.475 g
0.2% (w/v) CaCl ₂	0.40 g	0.50 g
0.1% (w/v) Tween 80	0.20 g	0.25 g
10 mM DTT	308.40 mg	385.5 mg
10% (w/v) PVPP (washed)	20 g	25 g
H ₂ O	to 200 ml	200 ml

- A. Dissolve the first 5 ingredients in H₂O and adjust the pH to 7.4. with NaOH. Store the solution at 4°C.
- B. Add the PVPP and DTT just before use.
- C. Shake well before using. The PVPP settles out quickly.

3) Column buffer

50 mM MOPS, pH 7.0	15.69 g	20.92 g
1.0 mM DTT	231.3 mg	308.4 mg
500 mM NaCl	43.83 g	58.44 g
0.1% (w/v) Tween 80	1.50 g	2.00 g
H ₂ O	to 1500 ml	2000 ml

- A. Prepare the solution without adding the DTT. Store at 4°C.
- B. Add the DTT as required (15.42 mg/100 ml) just before use.

4) DEAE Cellulose (See Himmelhoch, 1971)

- A. Use Whatman DE 52 microgranular pre-swollen DEAE cellulose.
- B. Weigh out the required amount of DEAE cellulose (30 g or less).
- C. Allow to sink into 1.0 M NaOH for 30 minutes at room temperature. Transfer to a Buchner funnel.
- D. Wash with water until the pH of the DEAE cellulose approaches neutrality. Use pH paper to monitor the pH.
- E. Resuspend in 0.5 M HCl, and filter through 500 ml of HCl.
- F. Wash with water until the pH again becomes neutral.
- G. Repeat the NaOH wash with 500 ml of 1.0 M NaOH.
- H. Wash out the alkali with water. Check pH.
- I. Wash the cellulose with the column buffer. Then, remove the DEAE cellulose from the funnel and resuspend in buffer in a beaker.
- J. Monitor the pH and adjust to 7.0 if necessary, using the pH meter.
- K. Return the DEAE cellulose to the funnel and wash with additional column buffer.
- L. Place the cake of DEAE cellulose into a bottle and store as a suspension in column buffer at 4°C. Add a bacteriostatic agent (0.02% thimerosal or 0.02% NaN₃ final concentration) to the suspension.

5) Gel stain

0.125% Coomassie Blue R-250	0.625 g
50% methanol	250 ml
10% acetic acid	50 ml
H ₂ O	to 500 ml

Be careful with Coomassie Blue. It will stain exposed skin, and is very difficult to wash off.

6) De-stain

40% methanol	400 ml
10% acetic acid	100 ml
H ₂ O	to 1000 ml

Procedure

Your T.A. will demonstrate how to assemble, pour and run your columns in the cold room

Pre-chill the mortar and pestle on ice. Prepare the extraction buffer. Chill 50 ml in a 100 ml beaker on ice.

A fine grayish powder forms. Work quickly.

Use a stirring plate set up in the cold room.

50 ml Corning orange cap tubes work well here. Mix everything by gentle inversion. Don't add the ammonium sulfate all at once.

The protein/polysaccharide pellet will coat the inside of the tube. The excess detergent forms an oily layer.

1. Prepare a 1 x 6 cm column of DEAE cellulose equilibrated to column buffer at 4°C. Set up 10% acrylamide, 0.1% SDS gels. Prepare a day ahead of time.

2. Prepare 5 g of Codium. Remove epiphytes. Rinse the Codium in distilled water. Blot dry.

3. Freeze the Codium in liquid nitrogen.

4. Grind the frozen Codium to a fine powder in a mortar and pestle. Add additional liquid nitrogen to the Codium as you grind. Keep the stuff frozen.

5. Transfer the frozen powder to 50 ml of ice-cold extraction buffer placed into a 100 ml beaker.

6. Mix (stir) for ²⁰~~10~~ minutes at 4°C.

7. Filter through 4 layers of Miracloth.

8. Divide the filtrate into two 30 ml Corex tubes and spin 5 Kg x 5 min at 4°C. *6.5 K rpm*

9. Carefully remove the supernatant. Measure its volume.

10. Add dry ammonium sulfate to 80% saturation (0.561 g/ml). Use the $(\text{NH}_4)_2\text{SO}_4$ chart. Add the $(\text{NH}_4)_2\text{SO}_4$ in thirds or fourths. Dissolve each addition before making another.

11. Transfer the solution to 50 ml polypropylene centrifuge tubes. Spin at 18.5 K rpm (40,000 x g) for 20 min, 4°C.

12. Carefully remove the supernatant. The proteins will coat the inside of the tube, which is very unusual. Examine the tube closely.

Your T.A. will provide some hints on how to do this.

Set the fraction collector to collect fractions of 80 drops each.

*run on large buffer
if green on top removed*

Watch out! Do not let your column run dry, or you will have to re-pack it.

Allow about 2 1/2 hours for the column to run.

There are several options available at this point. The method outlined here has worked, but it probably excludes some proteins. Your T.A. will be happy to discuss other options and the potential problems.

13. Resuspend your pellets in 1.0 ml of column buffer. Combine the pellets into one Eppendorf tube. Don't exceed 1.0 ml total volume.

14. Remove the column buffer from above the bed of DEAE cellulose in the column. Use a Pasteur pipet.

15. Carefully load the resuspended protein onto the column. Avoid disturbing the top of the bed.

16. Open the column outlet and allow the sample to run into the column. Begin collecting fractions at this point.

17. Wash the sample into the column with a few mls of column buffer. Fill the column with buffer and attach the reservoir. Adjust the flowrate to approximately 0.4 ml/min, or about 16 drops per minute.

18. Collect 20-30 fractions. Monitor the OD₂₈₀ for each fraction and plot the absorbance versus fraction number.

19. Remove 500 μ l from each peak fraction and place the samples into separate Eppendorf tubes. Add 1.0 ml of cold acetone, mix, and store at -20°C for at least one hour. Freeze the remaining fractions.

20. Pellet the precipitated protein in the microfuge at 4°C for 10 min. Pour off the supernatant, and remove the residual acetone.

21. Resuspend the pellets in about 125 μ l of sample buffer, and boil for 2 minutes.

22. Load about 30-40 μ l from each sample onto the gel, along with a track containing 2.5 μ g/band of molecular weight standards in 25 μ l of sample buffer. Electrophorese at 11-12 mamps per gel.

23. Stain the gel with Coomassie Blue. If the banding patterns look good, we will proceed with the immunoblots and lectin overlays. Otherwise, we need to discuss options.

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AMMONIUM SULFATE TABLE

This table indicates the correct amount of solid ammonium sulfate (at 25°C) to be added to one liter of solution to produce a desired change in the percent saturation of ammonium sulfate. Saturated ammonium sulfate at 25°C is 4.1M and requires 767 grams of salt per liter. "Per-

cent saturation" in this table is percent of 4.1M. The listed values were calculated from tables of percent salt, specific gravity and grams per liter at various concentrations.

Don't forget this chart

Per Cent Saturation
Final Concentration of Ammonium Sulfate

Per Cent Saturation Initial Concentration of Ammonium Sulfate	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
10		28	57	86	118	137	150	183	216	251	288	326	365	406	449	494	540	592	640	694
15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
33							12	43	74	107	142	177	214	252	292	333	379	426	472	522
35								31	63	94	129	164	200	238	278	319	364	411	457	506
40									31	63	97	132	168	205	245	285	328	375	420	469
45										32	65	99	134	171	210	250	293	339	383	431
50											33	66	101	137	176	214	256	302	345	392
55												33	67	103	141	179	220	264	307	353
60													34	89	105	143	183	227	269	314
65														34	70	107	147	190	232	275
70															35	72	111	153	194	237
75																36	74	115	155	198
80																	38	77	117	157
85																		39	77	118
90																			38	77
95																				39

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Unit 9.

Western blots: lectin overlays and immunoblotting

Introduction

Western blotting is the electrophoretic transfer of proteins from acrylamide gels onto nitrocellulose sheets or similar immobilizing matrices. The immobilized proteins, which represent a faithful replica of the protein banding pattern in the original gel, can now be readily examined by a wide variety of analytical techniques. We will concentrate on two analytical procedures, immunoblotting and lectin overlays.

In immunoblotting, western blots are treated with an antibody to a protein of interest. If the corresponding antigen is present on the nitrocellulose membrane, the antibody will bind to it. Similarly, lectins used to treat Western blots will bind to glycoproteins if the correct sugars are present. Lectins, such as Concanavalin A (Con A), soybean agglutinin (SBA) and ricin (RCA), have binding specificities for specific terminal sugars of the oligosaccharide moieties found on glycoproteins. Con A, for example, binds strongly to mannose, and has a lower affinity for glucose. The lectin in Codium fragile has a strong specificity for n-acetyl-galactosamine. Not only does a given lectin therefore indicate the glycoprotein nature of a given protein, but it also provides some information about monosaccharides comprising the oligosaccharides attached to that glycoprotein.

How, then, does one detect the presence of bound antibodies or lectins on Western blots? Several methods are available. Lectins (and proteins in general) may be labelled with Na¹²⁵I (iodinated) before treating the blot. After washing to remove unbound label, the ¹²⁵I incorporated into the bound lectin has sufficient energy to expose X-ray film placed against the blot. Results may be obtained in a few hours.

Most detection systems, however, rely on secondary binding reagents such as species-specific antibodies or Protein A, which in turn have attached a marker that permits visualization. These markers include colloidal gold, isotopes, or enzymes, usually horseradish peroxidase (HRP) or alkaline phosphatase (AP). HRP and AP convert a soluble substrate into a precipitating product, leaving a dark, visible band over the original protein on the Western blot. We will use HRP to detect the presence of the lectin Concanavalin A and the anti-Codium fragile lectin antibody.

HRP will be applied to blots previously treated with Con A. HRP is a glycoprotein itself, and hence will attach to any bound Con A. The HRP is then disclosed with the substrate 4-chloro-1-naphthol. Alternatively, Con A pre-labelled with HRP may be applied to the blot, thus eliminating the need for a secondary enzyme application step in the procedure.

Bound antibodies will be revealed with a second antibody conjugated with HRP. All of our antibodies were raised in rabbits. An HRP conjugated antibody to rabbit immunoglobulin raised in goats (goat anti-rabbit IgG) will bind to our antibodies which in turn are bound to the antigens on the nitrocellulose membrane. As before, the HRP will then be localized using 4-chloro-1-naphthol.

Areas of caution include the following:

1. Bear in mind the importance of setting up appropriate controls when preparing the original gels for Western blotting experiments. For example, always run a known glycoprotein that the lectin will bind to. If possible, run the antigen or a crude sample of the antigen to demonstrate the binding specificity of the antibody.

2. Do not touch nitrocellulose with bare fingers. Wear gloves, especially while mounting the gel for electrotransfer.
3. Avoid trapping bubbles between the gel and nitrocellulose when setting up your electrotransfers.
4. Nitrocellulose is highly flammable.
5. Lectins are toxic! Be careful with purified lectins. The purified lectin from castor beans, ricin, is one of the most toxic substances known.
6. 4-chloro-1-naphthol is a possible carcinogen. Handle this compound accordingly.

Western blotting

Materials

Fully electrophoresed PAGE gel
Pharmacia transfer apparatus
Nitrocellulose membrane
Metal edged ruler
Large pan or tray
Power supply

Solutions

- | | |
|--------------------|------------------------------|
| 1) Transfer buffer | |
| 20 mM Tris-base | Trizma base : 7.27g |
| 150 mM Glycine | Glycine : 33.80g |
| 0.1% SDS | SDS : 3.0g |
| 20% (v/v) Methanol | Methanol : 600ml |
| | H ₂ O to 3 liters |

Make two batches (6 liters total);

Dissolve the Tris (Trizma base), glycine and SDS in 1 liter of water final volume. Add the methanol, and additional water to make 3 liters. Store the solution in 4 liter containers.

Procedure:

The blue chromophore alters the electrophoretic mobility of the marker proteins. Consequently they should be used for rough estimation of molecular weights only.

Avoid touching the nitrocellulose with bare fingers. Use gloves, and keep the nitrocellulose between the protective blue backings.

Avoid trapping bubbles between the gel and the nitrocellulose. The bubbles will cause band distortion as the electrotransfer progresses.

This step can be difficult and frustrating. Relax, and repeat it as many times as necessary, until you feel comfortable with the set up.

1. When preparing the initial gel for electrophoresis, in addition to sample lanes, add a lane of BRL's pre-stained molecular weight markers. These markers have a covalently bound blue chromophore. This allows for visualization of the markers while the gel is running, and provides a quick way to judge the thoroughness of the electrotransfer.

Use about 10 μ l of markers per lane.

2. At the completion of electrophoresis, remove the gel from the glass plates and trim it to the desired size.

3. Measure the gel. Cut the nitrocellulose from the roll so that it exceeds the gel by about 0.5cm on all sides.

4. Soak the nitrocellulose in transfer buffer. Remove blue backings first.

5. Cut two pieces of Whatman 3 MM paper to match the size of the foam sponges in the transfer cassette. Soak the filter papers in transfer buffer.

6. In a tray of transfer buffer, place the nitrocellulose over a piece of filter paper. Float the gel over the nitrocellulose, and carefully push the gel down into position. Remove any trapped bubbles. Also, align the top of the separating gel with the top of the nitrocellulose.

7. Open up a transfer cassette. Set one of the plastic slides down and place a foam sponge over it.

8. Carefully lift the filter paper, nitrocellulose and gel from the pan and overlay it on the sponge. Avoid shifting the gel.

The orientation of the gel and nitrocellulose within the electric field is extremely important. We do not want to transfer the proteins in the wrong direction.

The proteins transfer from negative to positive; that is, from left to right in this arrangement.

9. Place the second piece of filter paper over the gel and follow that with the second sponge.

10. Snap the top of the transfer cassette into position. Pick up the cassette so that the nitrocellulose remains to the right of the gel.

11. Slide the cassette into the transfer box so that the nitrocellulose is to the right of the gel. Fill the box with transfer buffer (~ 4-5 liters).

12. Attach the positive electrode to the right, and the negative to the left.

13. Activate the power supply. Consult the transfer rig instructions for the proper voltage and time.

14. Remove the nitrocellulose from the cassette. Immediately mark the location of the pre-stained molecular weight standards with a waterproof pen. Although the markers are visible, they will fade occasionally.

15. Proceed with the lectin overlay or immunoblot procedures. Otherwise, dry the blot, and store it wrapped between filter paper sheets at 4°C indefinitely.

HRP-IgG Immunoblots

Materials

Western blotted proteins on nitrocellulose
Reaction tray
Shaker table
Imidazole
4-chloro-1-naphthol
H₂O₂
Antibodies
HRP-IgG

Solutions

1) TBS (Tris buffered saline)

50 mM Tris, pH 7.9
150 mM NaCl

10X stock solution
Tris: 30.28 g
NaCl: 43.83g
H₂O is 500 ml

Make a 10X stock solution. Adjust the pH to 7.9 with HCl. Dilute to 1X as required. (The concentrations above represent the 1X solution.)

2) TBST

50 mM Tris, pH 7.9
150 mM NaCl
0.05% (w/v) Tween 20

10X stock solution
Tris: 60.55 g
NaCl: 87.66 g
Tween 20: 0.5 g
H₂O to 1000 ml

Make up a 10X stock solution. Adjust pH to 7.9 with HCl. Dilute to 1X as required.

3) Blocking solution

0.5% BSA
0.02% NaN₃
in 1X TBST

1.0 g
40 mg
1X TBST to 200 ml

Dissolve the BSA carefully to avoid denaturing it.

4) Disclosing solution

Prepare this solution following the instructions below. See the chart for the amounts of reagents required.

- Prepare the appropriate volume of 0.01 M imidazole in 1X TBS.
- Prepare the corresponding amount of 4-chloro-1-naphthol at 3mg/ml in 100% methanol.
- Mix solutions (A) and (B).

- D. Add the indicated amount of H_2O_2 .
 E. Mix.
 F. Prepare immediately before use.

HRP disclosing solution

ml sol'n	ml TBS	mg imid.	ml MeO4	mg C-N	μ l H_2O_2
36	30	20.42	6.0	18.0	15
60	50	34.04	10.0	30.0	25
84	70	47.66	14.0	42.0	35

In general, add 2 ml of 4-chloro-1-naphthol (3 mg/ml) in MeOH to 10 ml of 0.01 M imidazole. Add 5 μ l of H_2O_2 to this mixture.

Note: H_2O_2 is 30% as supplied in the reagent bottle.

Procedure

The BSA and Tween 20 bind to the nitrocellulose, completely saturating the the unused protein sites and preventing the indiscriminate binding of the probes.

The blot may be stored in blocking solution for several days at 4°C.

There are several ways to do this. The TA will explain.

All of the remaining steps occur at room temperature.

Discard the HRP-IgG solution after one use.

1. Remove nitrocellulose blot from transfer rig and rinse in 1 X TBST, 3 times, 5 minutes each rinse.

2. Place nitrocellulose into the BSA blocking solution. Agitate for at least for at least 1 hour at room temperature with shaking.

3. Prepare the antibody solution. Make up 25 to 50 ml of 0.5 or 1.0% antibody in TBST. Add 0.02% (w/v) NaN_3 .

Reuse this solution several times. Store frozen when not in use.

4. Agitate the nitrocellulose in the antibody solution overnight at 4°C.

5. Next day. Wash the nitrocellulose 3 X 10 min in TBST at room temperature with shaking.

6. Remove the HRP conjugated goat anti-rabbit IgG from the refrigerator. Add 20 μ l to 50 ml TBST.

7. Treat the blot in GAR-IgG-HRP solution for 1-3 hr with shaking.

4-chloro-1-naphthol is light sensitive. Dim the lights or darken the room if possible. Do not agitate.

8. Wash the nitrocellulose 3 X 10 min with shaking. Use TBS (not TBST).

9. Pour the freshly made disclosing solution over the blot and watch carefully. Bands will begin to appear immediately in some cases, but usually within 3-4 minutes. Watch closely, especially for the development of background staining. If nothing develops within 15 minutes, give up.

10. Rinse the blot in distilled water to stop the disclosing reaction.

11. Dry the blot on filter paper and store it in the dark to prevent fading of the bands. Photograph the blot if a permanent record is required.

Concanavalin A - Horseradish peroxidase overlay of Western blots

This method, although specific for Con A, will work successfully for a variety of lectins. Some lectins require one or more bi-metallic cations to function properly. These cations must be changed and adjusted as required by the specific lectins. *Iodinate lectins otherwise*

Materials

Western blot with proteins
Shaker table
Reaction tray
Concanavalin A
Horseradish peroxidase
Con A labelled with HRP

Imidazole
4-chloro-1-naphthol
H₂O₂
 α -methyl-d-glucoside
Ovalbumin
Petri dishes

Solutions

1. Tris buffered saline (TBS)

40 mM Tris, pH 7.9
150 mM NaCl

10X stock solution
Tris : 30.28 g
NaCl : 43.83 g
H₂O to 500 ml

Adjust the pH to 7.9 with HCl. Dilute the 10X stock to 1X as required.

2. Stock solutions

A. 0.2 M MnCl₂

MnCl₂ : 1.98
H₂O to 50 ml

B. 0.2 M CaCl_2

CaCl_2 : 1.11 g
 H_2O to 50 ml

C. 0.2 M MgCl_2

MgCl_2 : 2.03 g
 H_2O to 50 ml

3. Blocking buffer

0.5% BSA	1.0 g
0.02% NaN_3	40 mg
in 1X TBS	1X TBS to 200 ml

Dissolve the BSA carefully to avoid denaturing it.

4. Wash buffer

10 μM CaCl_2	50 μl (0.2 M stock)
10 μM MnCl_2	50 μl (0.2 M stock)
10 μM MgCl_2	50 μl (0.2 M stock)
0.5% Triton X-100	5.0 g
1 X TBS	100 ml (10 X stock)
	H_2O to one liter

Dissolve the triton X-100 first in the TBS. Carefully add the salt solutions. MnCl_2 has a tendency to precipitate. Add this last.

Also note that Ca and Mn are absolutely essential for Concanavalin A to function.

5. Disclosing solution

Prepare the 4-chloro-1-naphthol disclosing solution as outlined in the immunoblot section.

Procedure

This experiment will use two controls. Ovalbumin is a known glycoprotein that Con A binds to. We will attempt to inhibit the binding of Con A to one of the duplicate samples by using the Con-specific sugar α -methyl-d-glucoside.

1. When preparing the SDS gel for this experiment, run two tracks of each protein sample to be probed, plus 2 tracks of ovalbumin (about 5 μg each well). For example

Well	Sample
1	Pre-stained markers
2	<u>Codium</u> extract
3	Ovalbumin - 5 μg
4	blank
5	blank
6	<u>Codium</u> extract
7	Ovalbumin - 5 μg

The blot may be stored in blocking buffer for several days at 4°C.

Be aware! Some commercial preparations of Con A are as much as 67% NaCl. Read labels, and weigh out more so that you do indeed have the correct amount of protein and not salt.

Expose the blot to Con A for as long as possible.

The α -methyl-d-glucoside is no longer necessary.

The longer these exposures the better. One hour each for Con A and peroxidase produces bands, but not very intense ones.

2. After the completion of the Western blot, separate the nitrocellulose into two sections between lanes 4 and 5.

3. Block the remaining unreacted protein binding sites by agitating the nitrocellulose in blocking buffer for at least one hour at room temperature.

4. (see step 6) Prepare a 1 mg/ml stock solution of Con A in wash buffer.

5. Dilute the stock solution to 10 μ g/ml in wash buffer. Prepare two solutions. To one, add α -methyl-d-glucoside at 40 mg/ml. Treat one half of the nitrocellulose with the α -methyl-d-glucoside solution, and the other half with the lectin solution not containing this sugar.

When properly trimmed, the nitrocellulose will fit neatly into a standard 100 mm petri dish. 7.5 ml of solution is sufficient to completely cover the nitrocellulose.

Agitate at least one hour at room temperature. Overnight agitation at 4°C is acceptable.

6. Alternatively, prepare a 1 mg/ml solution of Con A pre-labelled with peroxidase. Dilute this to 10 μ g/ml in wash buffer plus or minus 40 mg/ml α -methyl-d-glucoside. Agitate the duplicate halves plus or minus the α -methyl-d-glucoside overnight at 4°C.

Proceed with Step 10.

7. Wash the nitrocellulose in wash buffer: 5 X 5 minutes with agitation at room temperature.

8. Prepare a 50 μ g/ml solution of peroxidase in wash buffer (1.0 mg per 20 ml buffer).

9. Agitate the blot in the peroxidase solution for at least one hour at room temperature.

10. Wash the blots in wash buffer: 5
X 5 minutes with shaking.

11. Wash the blots twice in TBS, about
1-2 minutes each time.

12. Add the freshly made disclosing
solution and watch carefully for
bands. Bands should appear on the
section that was not exposed to the
 α -methyl-d-glucoside.

13. Rinse the blot in distilled
water. Dry, and store in the dark.

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Antibody overlay:

λ gt11 Library Immunoscreening Kit (CLIK) instructions. Clontech, Palo Alto, CA.